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(54) Title: MODULATION OF INSULIN-REGULATED AMINOPEPTIDASE (IRAP)/ANGIOTENSIN IV (AT₄) RECEPTOR ACTIVITY

(57) Abstract: The invention relates to modulators of insulin-regulated aminopeptidase (IRAP)/Angiotensin IV receptor (AT₄) activity, which have the ability to (a) increase local concentration of a wide range of peptides, thereby enhancing the activity of these peptides; (b) regulate glucose uptake into cells and tissues, thereby altering the metabolic or energy status of the cells or tissues; (c) trigger second messenger or signalling pathways associated with IRAP/AT₄ function; and/or (d) increase cell surface expression of IRAP/AT₄. The modulators may enhance learning and memory in both normal subjects and those with memory disorders. They also stimulate cellular proliferation while reducing apoptosis, thus stimulating tissue growth development, and affect angiogenesis. Thus they are also useful in tissue repair and regeneration. The invention also relates to methods of identifying modulators of insulin-regulated aminopeptidase activity.

Modulation of insulin- regulated aminopeptidase (IRAP)/Angiotensin IV (AT₄) receptor activity.

FIELD OF THE INVENTION

5 This invention relates to modulators of insulin-
regulated aminopeptidase activity, which have the ability
to increase local concentration of a wide range of peptide
hormones, thereby enhancing the activity of these
hormones. The modulators may enhance learning and memory
in both normal subjects and those with memory disorders.
10 They also stimulate cellular proliferation while reducing
apoptosis, thus stimulating tissue growth development, and
affect angiogenesis. Thus they are also useful in tissue
repair and regeneration. The invention also relates to
methods of identifying modulators of insulin-regulated
15 aminopeptidase activity.

BACKGROUND OF THE INVENTION

All references, including any patents or patent
applications, cited in this specification are hereby
20 incorporated by reference. No admission is made that any
reference constitutes prior art. The discussion of the
references states what their authors assert, and the
applicants reserve the right to challenge the accuracy and
pertinency of the cited documents. It will be clearly
25 understood that, although a number of prior art
publications are referred to herein, this reference does
not constitute an admission that any of these documents
forms part of the common general knowledge in the art, in
Australia or in any other country.

30 There are a number of conditions which have no
known treatment, or for which the treatments that are
available are not effective. For example, disorders of
the central nervous system, including Alzheimer's disease
(AD) and other forms of dementia and memory loss, motor
35 neurone diseases, disorders of the cardiovascular system,
including cardiac hypertrophy, congestive heart failure,
hypertension, and atherosclerosis, and disorders of

development and growth, including disorders of glucose and fat metabolism, are all serious conditions for which effective treatment is currently unavailable. Given the importance of these conditions, it is readily apparent
5 that effective treatments are urgently needed.

Alzheimer's disease (AD), for example, is one of the major health care problems facing first world countries. In 1995, 130,000 Australians aged over 65 years had moderate to severe dementia, and it is estimated
10 that by 2041 the number of people with dementia in Australia will have increased by 254% (Henderson and Jorm, 1998). Despite a dramatic increase in the understanding of the neuropathogenic and neurochemical alterations accompanying AD, physicians have as yet no pharmacological
15 agent that can be prescribed safely, either to arrest or reverse this condition.

Some of the other conditions identified above are also increasing in prevalence, and will become increasingly economically and socially burdensome.
20 Accordingly, there is a need for new forms of treatment and new therapeutic agents which can provide sufferers of these diseases with effective treatment.

Angiotensin IV (Ang IV) is a hexapeptide with the sequence VYIHPF (SEQ ID NO:1), which has been shown *in*
25 *vitro* to be produced by the consecutive actions of aminopeptidases A and N on angiotensin II. This hexapeptide was initially thought to be inactive, but has subsequently been shown to elicit actions in the brain and blood vessels. Ang IV acts via a specific binding site,
30 designated the Angiotensin IV receptor (AT₄ receptor).

The AT₄ receptor is defined as a high affinity binding site which selectively binds Ang IV with an affinity ranging from 1-10 nM (Swanson et al, 1992). The AT₄ receptor isolated from bovine adrenal membranes is a
35 glycoprotein of 165 kDa, with a single transmembrane domain, a large extracellular domain, and a small intracellular domain (Zhang et al, 1998). In the brain,

the AT₄ receptor occurs as a 150 kDa glycoprotein (Zhang et al, 1999). We have shown that a decapeptide, LVVYPWTQRF (SEQ ID NO:2) (Moeller et al, 1996) binds with nanomolar affinity to the AT₄ receptor, and can mimic the actions of
5 Ang IV, and that Nle¹-Ang IV (Nle¹-YIHPF) (SEQ ID NO:4) binds with sub-nanomolar affinity to AT₄ receptor (Zhang et al, 1999).

AT₄ receptors are widely distributed in the brains of guinea-pig (Miller-Wing et al, 1993), rat
10 (Roberts et al, 1995), sheep (Moeller et al, 1996), monkey (Moeller et al, 1996), and human (Chai et al, 2000), and the distributions of the receptors are highly conserved throughout these species. The receptor sites occur in high abundance in the basal nucleus of Meynert, in the CA2
15 and dentate gyrus of the hippocampus, and throughout the neocortex, a distribution which closely resembles those of cholinergic neurones and their projections, and is consistent with the memory-enhancing effects of the receptor. High levels of the receptors are also found in
20 most brain regions involved in motor control, including the motor cortex, ventral lateral thalamic nucleus, cerebellum and the cranial nerve motor nuclei. Many sensory regions also contain moderate levels of the AT₄ receptors, suggesting a more widespread role for this
25 receptor in the brain.

Central infusions of Ang IV, Nle-Ang IV and Norleucinal Ang IV, which are agonists of the AT₄ receptor, were found to facilitate memory retention and retrieval in rats in the passive avoidance and Morris water maze
30 paradigms (Braszko et al, 1988, Wright et al, 1993, Wright et al, 1999). In two rat models of amnesia, respectively induced by scopolamine or perforant pathway lesion, the AT₄ receptor agonists reversed the performance deficits detected in the Morris water maze paradigm (Pederson et
35 al, 1998; Wright et al, 1999). Ang IV was also reported to improve long-term memory in crabs (Delorenzi et al, 1999).

Insulin-regulated aminopeptidase (IRAP) is a constituent of vesicles containing the glucose transporter molecule GLUT4. See U.S. Patent No. 5,968,784 and No. 5,972,600, both by Knowles et al. It is known that the distribution and recycling of both IRAP and GLUT4 between cellular vesicles and the cell membrane is regulated by insulin signalling, and thereby modulates glucose, fat and energy metabolism in tissues such as muscle, adipose tissue, and cardiac tissue (Waters et al, 1997, Ross et al, 1998, Garza and Birnbaum, 2000). However, it has never to our knowledge previously been suggested that IRAP might have any role in central nervous system function, and more specifically it has not been suggested that this protein has any role in memory or learning.

We have now isolated the AT₄ receptor by protein purification, and have determined its amino acid sequence. We surprisingly discovered that the AT₄ receptor has a span of 18 amino acid residues which has a 95% sequence homology with the deduced amino acid sequence of human oxytocinase (TrEMBL accession # 000769), which is known to be a homologue of insulin-regulated aminopeptidase (IRAP) (Rasmussen et al, 2000). This enabled us to demonstrate that IRAP is the AT₄ receptor, and to hypothesise that modulation of the activity of IRAP by peptides such as Ang IV, Nle¹-Ang IV, LVVYPWTQRF (SEQ ID NO:2) and KYLPGLQ (SEQ ID NO:3) will activate neurones in the central nervous system to enhance learning and memory. Moreover, the identification of IRAP as the AT₄ receptor means that screening assays can be developed to identify compounds which have the ability to modulate IRAP enzymatic activity. Such agents can be used to increase serum and tissue levels of biologically active compounds such as arginine-vasopressin, oxytocin, somatostatin, angiotensin III, Lys-bradykinin, neurotensin, met-enkephalin, dynorphin A, neurokinin A, neuromedin B, cholecystokinin 8, insulin, insulin-like growth factor 1 or insulin-like growth factor 2 which may assist in preventing or treating

conditions associated with deficient activity of these compounds.

SUMMARY OF THE INVENTION

- 5 In a first aspect, the invention provides a method of screening for compounds which can modulate the activity of AT₄ receptor/insulin-regulated aminopeptidase (IRAP), comprising the step of
- 10 a) assessing the ability of a candidate compound to bind to AT₄ receptor/IRAP and/or to increase or decrease the enzymatic activity of AT₄ receptor/IRAP *in vitro*, and optionally also comprising the step of
- 15 b) assessing the effect of the candidate compound on the interaction between AT₄ receptor/IRAP and a protein which interacts with AT₄ receptor/IRAP, such as tankyrase.
- In preferred embodiment, this aspect provides:
1. A method comprising the steps of
- (a) assessing the ability of the candidate compound to bind to AT₄ receptor/IRAP, and
- 20 (b) assessing the ability of the candidate compound to modulate enzymatic activity of AT₄ receptor/IRAP, thereby to identify compounds which bind to AT₄ receptor/IRAP with high affinity and modulate its enzymatic activity;
- 25 2. A method comprising the steps of
- (a) assessing the ability of the candidate compound to bind to AT₄ receptor/IRAP and
- (b) assessing the ability of the candidate compound to modulate one or more signalling or intracellular
- 30 pathways activated by binding of the candidate compound to AT₄ receptor/IRAP, thereby to identify compounds which bind to AT₄ receptor/IRAP with high affinity and modulate its signalling/second messenger activity;
- 35 3. A method comprising the steps of
- (a) assessing the ability of the candidate compound to bind to AT₄ receptor/IRAP and

- (b) assessing the ability of the candidate compound to modulate activity of AT₄ receptor/IRAP, thereby to identify compounds which bind to AT₄ receptor/IRAP with high affinity and modulate other cellular activity associated with AT₄ receptor/IRAP;
- 5 4. A method comprising the steps of
- (a) identifying candidate proteins which interact with AT₄ receptor/IRAP and
- (b) assessing the ability of the proteins to modify
- 10 the enzymatic or signalling activities of AT₄ receptor/IRAP, thereby to identify intracellular or extracellular proteins which interact with AT₄ receptor/IRAP and modulate its activity; and
- 15 5. A method comprising the steps of assessing the effect of the candidate compound on
- (a) binding to AT₄ receptor/IRAP or to a protein which interacts with AT₄ receptor/IRAP, or
- (b) altering the ability of proteins to interact with
- 20 AT₄ receptor/IRAP, and on
- (c) modifying the enzymatic or signalling activities of AT₄ receptor/IRAP, thereby to identify compounds which bind to AT₄ receptor/IRAP or to a protein which interacts with AT₄
- 25 receptor/IRAP, and modifies the activity of AT₄ receptor/IRAP.

In a second aspect, the invention provides a method for modulating the expression, production, or activity of AT₄ receptor/IRAP, comprising the step of

30 administering an effective amount of a compound which alters AT₄ receptor/IRAP expression, production or activity to a subject in need of such treatment, in which the compound is a specific, high affinity ligand of AT₄ receptor/IRAP which modulates AT₄ receptor/IRAP levels or

35 AT₄ receptor/IRAP activity.

In a preferred embodiment, the invention provides a method of modulating the *in vivo* concentration of a

neuropeptide and/or a polypeptide, comprising the step of administering an effective amount of a compound which can modulate the activity of AT₄ receptor/IRAP to a subject in need of such treatment.

5 Preferably the neuropeptide is selected from the group consisting of arginine-vasopressin, oxytocin, somatostatin, angiotensin III, Lys-bradykinin, neurotensin, met-enkephalin, dynorphin A, neurokinin A, neuromedin B, and cholecystokinin 8. Preferably the
10 polypeptide is selected from the group consisting of insulin, insulin-like growth factor I and insulin-like growth factor II. Cholecystokinin 8 is an 8-amino acid peptide derived from cholecystokinin, which is known to have activity in the brain.

15 This aspect of the invention is generally directed to a method of prevention or treatment of a disease condition associated with altered activity of AT₄receptor/IRAP, comprising the step of administering an effective amount of a compound which is able to modulate
20 the activity of AT₄ receptor/IRAP to a subject in need of such treatment. Alternatively, this aspect is directed to a method for the prevention or treatment of a disease or condition by altering the activity of AT₄ receptor/IRAP, comprising the step of administration of an effective
25 amount of a compound which has the ability to modulate the activity of AT₄ receptor/IRAP to a subject in need of such treatment.

 Compounds which modulate IRAP activity may be used to treat or prevent disorders of the central nervous
30 system (CNS), including Alzheimer's disease and other forms of dementia and memory loss; other disorders of the CNS, particularly those involving motor and sensory systems; disorders of the CNS resulting from trauma or stroke; disorders of the cardiovascular system, including
35 cardiac hypertrophy, congestive heart failure, vasospastic disorders, hypertension, and atherosclerosis; disorders of development and growth; disorders of the reproductive

tract or disorders associated with pregnancy; disorders of glucose and fat metabolism including syndrome X, dyslipidaemia, and diabetes; and cancer; and for promotion of neuroregeneration and repair following CNS damage or injury, promotion of tissue regeneration and repair following damage or injury, promotion of wound healing, inhibition of angiogenesis, and for treatment of premature or delayed labour.

It is therefore contemplated that the method of the invention is suitable for treatment of a variety of conditions, including but not limited to neurodegenerative diseases, such as motor neurone disease (amyotrophic lateral sclerosis), progressive spinal muscular atrophy, infantile muscular atrophy, Charcot-Marie-Tooth disease, Parkinson's Disease, Parkinson-Plus syndrome, Guamanian Parkinsonian dementia complex, progressive bulbar atrophy, Alzheimer's disease and the like; other neurodegenerative conditions, such as those arising from ischaemia, hypoxia, neural injury, surgery, or exposure to neurotoxins such as N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine); and peripheral sensory neuropathies, including those resulting from exposure to drugs such as cis-platin or toxins, and those resulting from diabetes, for example mononeuropathy multiplex.

In one preferred embodiment, the invention provides a method of preventing or treating a condition characterised by neuronal deficit or neuronal death, comprising the step of administering an effective amount of a compound or agent of the invention to a subject in need of such treatment.

In a second preferred embodiment, the invention provides a method of stimulation of growth in a mammal, comprising the step of administering an effective amount of a compound which can modulate the activity of AT_4 receptor/IRAP to the mammal. This embodiment is particularly applicable to farm mammals such as a sheep, cattle, pigs and goats.

Preferably the compounds which modulate AT₄ receptor/IRAP activity are able to decrease or increase the enzymatic activity of AT₄ receptor/IRAP, thereby increasing the serum and tissue levels of biologically-active arginine-vasopressin, oxytocin, somatostatin, angiotensin III, Lys-bradykinin, neurotensin, met-enkephalin, dynorphin A, neurokinin A, neuromedin B, cholecystokinin 8, and/or insulin.

Persons skilled in the art will appreciate that exogenous administration of a compound identified by the methods of this invention would have similar effects and uses to administration of exogenous arginine-vasopressin, oxytocin, somatostatin, angiotensin III, Lys-bradykinin, neurotensin, met-enkephalin, dynorphin A, neurokinin A, neuromedin B, cholecystokinin 8, insulin, IGF-I or IGF-II.

In a third aspect, the invention provides a method of enhancing memory and/or learning in a normal subject, comprising the step of administering an effective amount of a compound which can modulate the activity of AT₄ receptor/IRAP to a subject in need of such treatment. Alternatively, this aspect provides a method of treating memory disorders and/or of reversing memory loss, comprising the step of administration of an effective amount of a compound which has the ability to modulate the activity of AT₄ receptor/IRAP to a subject in need of such treatment.

In a fourth aspect, the present invention provides a method of monitoring the activity of AT₄ receptor/IRAP, comprising the step of assessing the aminopeptidase activity of AT₄ receptor/IRAP in a biological sample. The biological sample is preferably a biological fluid such as blood, plasma, cerebrospinal fluid, lymph, amniotic fluid or urine, but may also be a tissue sample or a sample of tissue or cell culture medium.

In a fifth aspect, the invention provides a method of diagnosing an AT₄ receptor/IRAP-related

condition, comprising the step of measuring the level of activity of AT₄ receptor/IRAP in a subject suspected of having such a condition, and comparing the level to that found in a normal individual, in which any difference
5 between the levels is indicative of the condition. The activity of AT₄ receptor/IRAP may be measured in a sample of a biological fluid such as blood, serum, plasma, lymph, amniotic fluid or cerebrospinal fluid, or in a tissue or cell sample.

10 In a sixth aspect, the invention provides a therapeutic or prophylactic agent comprising
(a) a peptide which has an amino acid sequence selected from the group consisting of LVVYPWTQRF (SEQ ID NO:2), KYLPGPLQ (SEQ ID NO:3) and VYIHPF (SEQ ID NO:1), or
15 an analogue or peptidomimetic thereof which is able to modulate the activity of AT₄ receptor/IRAP,

(b) an inhibitor of leucine aminopeptidase activity, such as L-leucinethiol, L-leucinal, L-leucinol, or derivatives thereof which have the ability to inhibit
20 IRAP, or

(c) an antisense AT₄ receptor/IRAP nucleic acid molecule or a fragment thereof which has the ability to inhibit expression of AT₄ receptor/IRAP, and optionally

(d) a pharmaceutically acceptable carrier.
25 Variant sequences of these peptides, for example encompassing short deletions or conservative substitutions of amino acids, are within the scope of the invention, provided that the ability to modulate the activity of AT₄ receptor/IRAP is retained.

30 It is to be clearly understood that the compounds of the invention include peptide analogues, including but not limited to the following:

1. Compounds in which one or more amino acids is replaced by its corresponding D-amino acid. The skilled
35 person will be aware that retro-inverso amino acid sequences can be synthesised by standard methods. See for example Chorev and Goodman, 1993;

2. Peptidomimetic compounds, in which the peptide bond is replaced by a structure more resistant to metabolic degradation. See for example Olson et al, 1993; or

5 3. Compounds in which individual amino acids are replaced by analogous structures, for example *gem*-diaminoalkyl groups or alkylmalonyl groups, with or without modified termini or alkyl, acyl or amine substitutions to modify their charge.

10 The use of such alternative structures can provide significantly longer half-life in the body, since they are more resistant to breakdown under physiological conditions.

Methods for combinatorial synthesis of peptide
15 analogues and for screening of peptides and peptide analogues are well known in the art (see for example Gallop et al, 1994). It is particularly contemplated that the compounds of the invention are useful as templates for design and synthesis of compounds of improved activity,
20 stability and bioavailability.

Preferably where amino acid substitution is used, the substitution is conservative, i.e. an amino acid is replaced by one of similar size and with similar charge properties.

25 In a seventh aspect, the invention provides a method of modulating the activity of AT₄ receptor/IRAP, comprising the step of administering to a subject in need thereof an effective amount of an antisense nucleic acid molecule directed against nucleic acid encoding AT₄
30 receptor/IRAP, or a fragment thereof which has the ability to inhibit expression of AT₄ receptor/IRAP.

Preferably, but not exclusively, the antisense AT₄ receptor/IRAP nucleic acid molecule has a nucleotide sequence selected from the group consisting of SEQ ID
35 NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID

NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22 and SEQ ID NO:23.

5 The antisense oligonucleotide may optionally be operatively linked to a vector and/or may be present in a transfected host cell.

In an eighth aspect, the invention provides an oligonucleotide molecule which is antisense to a nucleic acid encoding AT₄ receptor/IRAP, which is

- 10 a) a nucleotide sequence selected from the group consisting of SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10; SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, 15 SEQ ID NO:22 and SEQ ID NO:23;
- b) a fragment of a) which has the ability to inhibit expression of AT₄ receptor/IRAP;
- c) a nucleic acid molecule which has at least 75% sequence homology to a sequence in a) or b);
- 20 d) a nucleic acid molecule which is capable of hybridizing to a sequence in a) or b) under stringent conditions; or
- e) a nucleic acid molecule of homologous sequence derived from the species listed below.

25 In one embodiment, the invention provides a construct for delivering *in vivo* an effective amount of an antisense nucleic acid molecule directed against nucleic acid encoding AT₄ receptor/IRAP, comprising:

- 30 a) an oligonucleotide as in a) above; and
 - b) a vector comprising a control sequence,
- wherein the control sequence is capable of controlling the expression of a nucleotide sequence of a), which in turns modulates the activity of AT₄ receptor/IRAP.

35 Modified and variant forms of the constructs disclosed herein may be produced *in vitro*, by means of chemical or enzymatic treatment, or *in vivo* by means of recombinant DNA technology. Such constructs may differ

from those disclosed, for example by virtue of one or more nucleotide substitutions, deletions or insertions, but substantially retain a biological activity of the construct or nucleic acid molecule of this invention.

5 In a ninth aspect, the invention provides a composition comprising one or more agents or antisense oligonucleotides according to the invention, together with a pharmaceutically acceptable carrier.

The compositions of the invention may be
10 formulated so as to be suitable for a variety of routes of administration, for example intravenous, subcutaneous, intramuscular or intrathecal or intraventricular injection, or for oral or topical administration.

The exact formulation will depend on the
15 individual route of administration. Methods and pharmaceutical carriers for preparation of pharmaceutical compositions are well known in the art, as set out in textbooks such as Remington's Pharmaceutical Sciences, 17th Edition, Mack Publishing Company, Easton, Pennsylvania,
20 USA. Pharmaceutically acceptable carriers include conventional carriers which are suitable for use with peptide-based drugs, including diluents, excipients, and preservatives and the like. For example, carriers such as dextrose, mannitol, sucrose, or lactose, buffer systems
25 such as acetate, citrate and phosphate, and bulking agents such as serum albumin, preferably human serum albumin, may be used.

Preferably the antisense oligonucleotide is presented as a composition, which may be in the form of a
30 capsule or a cartridge, or as a kit provided with a delivery device and instructions for its use. The kit may also include other therapeutic agents, and ingredients for the composition.

The dose required will depend on the nature and
35 severity of the condition to be treated, and the route of administration, and will be at the discretion of the attending physician or surgeon. A suitable route,

frequency of administration, and dosage can readily be established using conventional clinical trial methodology.

While it is particularly contemplated that the methods and compounds of the invention are suitable for use in the medical treatment of humans, they are also applicable to veterinary treatment, including treatment of companion animals such as dogs and cats, and domestic animals such as horses, cattle and sheep, or zoo animals such as non-human primates, felids, canids, bovids, and ungulates.

Methods and pharmaceutical carriers for preparation of pharmaceutical compositions are well known in the art, as set out in textbooks such as Remington's Pharmaceutical Sciences, 19th Edition, Mack Publishing Company, Easton, Pennsylvania, USA.

The compounds and compositions of the invention may be administered by any suitable route, and the person skilled in the art will readily be able to determine the most suitable route and dose for the condition to be treated. Dosage will be at the discretion of the attendant physician or veterinarian, and will depend on the nature and state of the condition to be treated, the age and general state of health of the subject to be treated, the route of administration, and any previous treatment which may have been administered.

The carrier or diluent, and other excipients, will depend on the route of administration, and again the person skilled in the art will readily be able to determine the most suitable formulation for each particular case.

The methods and compositions of the invention may be used in conjunction with, ie before, during and after one or more other treatments for the condition concerned. The agent may be administered prophylactically or therapeutically, and may be used either alone or in conjunction with other therapies for conditions without known therapies, or as a substitute for therapies that

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have significant negative side effects.

The composition of the invention may be administered by any suitable route, including oral, intracavitary, intranasal, intraanal, intravaginal, transdermal, intradermal, buccal, intravenous, subcutaneous, intramuscular, by inhalation, intraglandular, intraarterial, intravascular in general, into the ear, intracranial, intrathecal, intraorgan, by implantation, and intraocular administration.

For the purposes of this specification it will be clearly understood that the word "comprising" means "including but not limited to", and that the word "comprises" has a corresponding meaning.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows non-reducing SDS-PAGE analysis of fractions from DEAE-Sepharose ion exchange purification of the AT₄ receptor. The gel was silver stained (A) and subjected to autoradiography (B). Proteins from bovine adrenal membranes, solubilised with CHAPS, were fractionated on a DEAE-Sepharose fast flow column. A silver-stained protein band (indicated by an arrowhead), which co-migrated with the radiolabelled band, was detected in eluted fractions, as shown in lane 2. Molecular weight markers are shown in lane 1, with the sizes in kDa indicated to the left of the figure.

Figure 2 shows the results of capillary column RP-HPLC/ESI-MS/MS analysis of the silver-stained protein band shown in Figure 1 at M_r 165kD.

(A) MS/MS total ion current (TIC) trace of the tryptic digest. The peptide mixture was applied to a 0.2 mm ID column and a 60 min linear gradient (flow rate 1.7 μ L/min) was used from 0-100% B, in which solvent A was 0.1% v/v aqueous TFA, and solvent B was 0.1% v/v aqueous TFA in 60% acetonitrile. Peptides were sequenced by collision-induced dissociation (CID) of their parent ions. Peaks labelled with an asterisk were identified as trypsin

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auto-digestion peptides. Peptides T_1 and T_2 were identified using the SEQUEST algorithm as residues 321-349 and 208-220 respectively, from *Bos taurus* CD34 (TrEMBL accession number Q9XS61).

- 5 (B) Collision-induced dissociation MS/MS spectrum of the doubly-charged ion of tryptic peptide T_3 (m/z 1106.0). Peptide T_3 could not be identified using SEQUEST, and was interpreted using *de novo* methods, i.e., manual interpretation of the β - and γ -type product ion series
10 (Roepstorff and Fohlman, 1984), as shown. The sequence tag VQAFFE was deduced first, and this was searched against the TrEMBL database using the Fasta program. This sequence tag was found to be homologous to human oxytocinase (TrEMBL accession number 000769). Further
15 residues from human oxytocinase (residue numbers 979-996) were found to fit the MS/MS spectrum.

- (C) The sequence of the 19 amino acid peptide thus identified is shown. It differs from homologous region of human oxytocinase only in the N-terminal residue, which is
20 a threonine in the human sequence.

- Figure 3 summarises results from two binding studies, showing an increase in ^{125}I -Nle¹ Ang IV binding to membranes prepared from HEK 293 cells which have been transfected with plasmids containing the IRAP cDNA (IRAP)
25 compared to membranes from HEK 293 cells transfected with control plasmids (control). The binding of the radioligand is competitively inhibited by increasing concentrations of unlabelled Ang IV. Bo represents the total number of ^{125}I -Nle¹ Ang IV binding sites in the IRAP
30 transfected cells, and $B/\text{Bo} \times 100$ represents the number of binding sites occupied, expressed as a percentage of the total number of sites in the IRAP transfected cells. Pci IRAP was prepared as described by Rogi et al, 1996.

- Figure 4 summarises results from a binding study
35 showing an increase in ^{125}I -Nle¹ Ang IV binding to membranes prepared from HEK 293 cells transfected with plasmids containing the IRAP cDNA (IRAP), compared to membranes

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from HEK 293 cells transfected with control plasmids (control). The binding of the radioligand is competitively inhibited by increasing concentrations of unlabelled LVVYPWTQRF (SEQ ID NO:2), a known AT₄ receptor ligand. Bo represents the total number of ¹²⁵I-Nle¹ Ang IV binding sites in the IRAP transfected cells, and B/Bo x 100 represents the number of binding sites occupied, expressed as a percentage of the total number of sites in the IRAP transfected cells.

Figure 5 summarises results from a binding study showing an increase in ¹²⁵I-Nle¹ Ang IV binding to membranes prepared from HEK 293 cells transfected with plasmids containing the IRAP cDNA (IRAP) compared to membranes from HEK 293 cells transfected with control plasmids (control). The binding of the radioligand is competitively inhibited by increasing concentrations of unlabelled KYLPGPLQ (SEQ ID NO:3), a known AT₄ receptor ligand. Bo represents the total number of ¹²⁵I-Nle¹ Ang IV binding sites in the IRAP transfected cells and B/Bo x 100 represents the number of binding sites occupied, expressed as a percentage of the total number of sites in the IRAP transfected cells.

Figure 6 shows an autoradiograph of non-reducing SDS-PAGE analysis of [¹²⁵I]-Nle¹-BzPhe⁶-Gly⁷-Ang IV cross-linked AT₄ receptor/IRAPs from membranes from HEK 293-T cells transfected with IRAP cDNA (IRAP), compared to membranes from HEK 293-T cells transfected with control plasmids (control), showing total (T) and non-specific (NS) binding. Transfected cells were solubilised in 1% CHAPS, 50 mM Tris, pH 6.8, 5 mM EDTA for 4 h at 4°C. Membranes were removed by centrifugation, and the solubilised receptor incubated with 0.1 µCi [¹²⁵I]-Nle¹-BzPhe⁶-Gly⁷-Ang IV, 20 µM bestatin, 100 µM PMSF, 2.5 mM phenanthroline, in a total volume of 100 µl for 90 min at 37°C, then UV irradiated at 365 nm for 2 h on ice. Two specific radiolabelled bands are visible in cells transfected with IRAP cDNA; a major band of approximately 165kDa and a minor band of >250 kDa.

Figure 7 demonstrates the distribution of AT₄ receptor binding using ¹²⁵I-Nle Ang IV binding in a mouse brain section and IRAP mRNA by *in situ* hybridization histochemistry, in an adjacent section.

5 Figure 8 illustrates the distribution of AT₄ receptor binding in E15 mouse embryo using ¹²⁵I-Nle Ang IV and IRAP mRNA using antisense oligonucleotides.

10 Figure 9 demonstrates the distribution of IRAP mRNA in human cerebellar sections by *in situ* hybridization histochemistry, using antisense and sense oligonucleotide sequences derived from the human IRAP cDNA sequence (Top panels). The antisense sequences used include nucleotides 1136-1109, nucleotides 1407-1377 and nucleotides 2587-2560 of the human IRAP sequence (Human Genbank Acc.U62768). The
15 adjacent sections of the cerebellum were labelled with ¹²⁵I-Nle Ang IV to demonstrate AT₄ receptor binding (Bottom panel).

20 Figure 10 shows an autoradiograph of non-reducing SDS-PAGE analysis of [¹²⁵I]-Nle¹-BzPhe⁶-Gly⁷-Ang IV cross-linked AT₄ receptor/IRAPs from bovine adrenal membranes, purified by DEAE-Sepharose anion exchange chromatography. A major band with apparent molecular weight of 165 kDa is visible, as well as three higher molecular weight complexes (indicated by arrowheads).

25 Figure 11 shows an autoradiograph of SDS-PAGE analysis of [¹²⁵I]-Nle¹-BzPhe⁶-Gly⁷-Ang IV cross-linked AT₄ receptors from SK-N-MC cells (A) and bovine adrenal membranes (B). Shown are total [¹²⁵I]-Nle¹-BzPhe⁶-Gly⁷-Ang IV binding to SK-N-MC and bovine adrenal membranes under
30 non-reducing conditions (Lane 1) and reducing conditions (Lane 2), in the presence of 10 μM unlabelled Ang IV under non-reducing (Lane 3) and reducing conditions (Lane 4) and in the presence of 10 μM unlabelled LVVYPWTQRF (SEQ ID NO:2) under non-reducing (Lane 5) and reducing conditions
35 (Lane 6). A minor higher molecular weight radiolabelled complex, which migrates with an apparent molecular weight of approximately 250 kDa, is apparent under non-reducing

conditions (Lane 1), but not under reducing conditions (Lane 2).

Figure 12a shows the results of measurement of inhibition of IRAP activity by Ang IV and LVVYPWTQRF (SEQ ID NO:2), using a fluorescence assay with the synthetic substrate L-leucyl- β -naphthylamide. The rate of hydrolysis of L-leucyl- β -naphthylamide by IRAP in the presence of 10 mM Ang IV or LVVYPWTQRF (SEQ ID NO:2) (LVV-hemorphin 7) was detected by the change in fluorescence at an excitation wavelength of 340 nm and an emission wavelength of 410 nm.

Figure 12b illustrates the kinetic analysis of IRAP inhibition. Double-reciprocal (Lineweaver-Burk) plots of IRAP activity in the absence and presence of three concentrations of Ang IV (Top panel) or divalinal Ang IV (Bottom panel) are shown.

Figure 13 shows the amino acid residues of the three peptides which bind with high affinity to the AT₄ receptor/IRAP aligned at the tyrosine residue. Structure-activity studies on the longest peptide

LVVYPWTQRF (SEQ ID NO:2) revealed that the residues VYPWT still retained full AT₄ receptor/IRAP binding activity.

Figure 14a shows a summary of the effect of Nle Ang IV in the Barnes Maze spatial learning paradigm. The upper panel is a plot of the mean error of three treatment groups given artificial cerebrospinal fluid control, Nle Ang IV at 1 nmol or Nle Ang IV at 100 pmol respectively. The lower panel is a plot of the number of days taken by the three treatment groups reach learner criteria.

Figure 14b demonstrates the effect of AT₄ ligands, Nle-Ang IV and LVVYPWTQRF (SEQ ID NO:2), to accelerate learning in the Barnes circular maze paradigm. The top panel illustrates the learner curves, which are

plots of the percentage of animals reaching learner criteria over the days of testing for the two peptide treatments. The bottom panel are plots of the mean number of days the animals take to reach learner criteria after a single dose of either peptide 5 min before the first trial on the first day.

Figure 15 summarises the effect of chronic infusion of LVVYPWTQRF (SEQ ID NO:2) at a rate of 0.3 pmol/h on the Y maze spatial learning paradigm, after 3 and 6 days in mice. The plot is of the % time spent in the novel arm of the Y maze. Increased time spent in the novel arm reflects improved memory retention as a result of the treatment.

Figure 16 summarises the effect of an acute intracerebroventricular (icv) dose of LVVYPWTQRF (SEQ ID NO:2) on the amnesic effect of scopolamine treatment in the Morris Water Maze paradigm in rats. The latency in time in finding the submerged platform is a measure of the amnesic property of the different treatments.

Figure 17 summarises the effect of an acute (icv) dose of LVVYPWTQRF (SEQ ID NO:2) on the amnesic effect of scopolamine treatment in the passive avoidance paradigm in rats. The latency in time in entering the dark chamber is a measure of the memory-enhancing property of the different treatments.

Figure 18 demonstrates the effect of Ang IV (A) and LVVYPWTQRF (SEQ ID NO:2) (B) on potassium-evoked ^3H -choline release from rat hippocampal slices, using 10^{-9} to 10^{-6} M of each peptide. Values are the mean \pm SEM of six experiments at each dose, and are expressed as a percentage of control. * $p < 0.05$.

Figure 19a illustrates stimulation of ERK1 and ERK2 phosphorylation in rat hippocampal slices treated with 10^{-8} M Nle-Ang IV in aCSF, for 30 min, aCSF alone or 30 mM KCl and 5 mM forskolin for 10 min.

Figure 19b shows the effect of three different concentrations (0.001, 1 and 10 μM) of Nle-Ang IV on

ERK1/ERK2 phosphorylation in HEK 293 cells transfected with human IRAP treated for 5 min, 30 min and 120 min with the peptide.

Figure 20 shows a section of mouse hippocampus stained with an antibody directed against the cytoplasmic tail of murine IRAP. The IRAP antibody staining was detected in the pyramidal cell layer of CA3 region of Ammon's horn (top panel - right), corresponding to neurones demonstrated by double labelling with the neuronal marker NeuN antibody (top panel - left). The bottom panel shows that IRAP (left) was found in the same neurones as GLUT4 (right) but not in the same sub-cellular compartments.

Figure 21 illustrates stimulation of [³H]-thymidine incorporation in NG108-15 or SK-N-MC cells following exposure to 10⁻¹⁰ to 10⁻⁶ M LVVYPWTQRF (SEQ ID NO:2). Values are the mean ± SEM of several experiments performed in quadruplicate, and are expressed as a percentage of control. *p<0.05.

Figure 22 summarises the effect of LVVYPWTQRF (SEQ ID NO:2) and Ang IV on neurite outgrowth from E15 chick sympathetic neurones. The values are expressed as % of control.

Figure 23 shows the distribution of AT₄ receptor/IRAP in the developing mouse brain, from embryonic day 13.5 to 17. The sections were cut in the sagittal plane.

Figure 24 illustrates [¹²⁵I]-Ang IV binding to a section of damaged sheep spinal cord. The arrowheads indicate binding to blood vessels, and the arrows indicate activated glia. Scale bar = 1.9 mm.

Figure 25 illustrates [¹²⁵I]-Ang IV binding to an uninjured rabbit carotid artery. The left figure is a haematoxylin and eosin-stained section. The right figure represents a haematoxylin and eosin-stained section which has been overlaid with the corresponding X-ray film

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exposed to the incubated section. e=endothelial cells, m=media, a=adventitia. Scale bar=0.15mm.

Figure 26 represents the results of quantification of [125 I]- Ang IV binding in the media/neointima of the right carotid artery of rabbits, (A) 1 day (1di), (B) 1 week (1wi), (C) 4 weeks (4wi) and (D) 20 weeks (20wi) after balloon injury. Values are the mean \pm SEM of 3-4 animals, and are expressed as a percentage of control tissues (the left carotid arteries): 1 day untreated (1du), 1 week untreated (1wu), 4 weeks untreated (4wu) and 20 weeks untreated (20wu) * $p < 0.05$.

Figure 27 shows the stimulation of cell surface expression of IRAP by serum in SN56 and SK-N-MC cell lines detected by an ELISA assay. Results are expressed as percent of basal (Top panel). Presence of IRAP (arrowhead) in high density microsomes (2), low density microsomes (3) and plasma membrane (4) in SK-N-MC cells after serum stimulation detected by Western blot analysis. Note its absence in cytoplasm (1) and nucleus and mitochondria (5) (bottom panel).

DETAILED DESCRIPTION OF THE INVENTION

The invention will now be described in detail by way of reference only to the following non-limiting examples and drawings.

We have purified the AT_4 receptor by anion exchange and wheat germ lectin affinity chromatography, and determined its amino acid sequence by tandem mass spectrometry of tryptic peptides. A search of the EMBL protein sequence database found that 18 of the 19 amino acid residues comprising the derived sequence were identical to residues 978-996 of human oxytocinase (TrEMBL accession # O00769), which is the homologue of insulin-regulated aminopeptidase (IRAP).

To confirm that IRAP is indeed the AT_4 receptor, we have transfected expression plasmids containing the IRAP cDNA into HEK 293 cells and Chinese hamster ovary

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(CHO) cells, and found a 10-20 fold increase in sites which specifically bind ^{125}I -Nle¹ Ang IV. The binding of ^{125}I -Nle¹ angiotensin IV to these sites can be competitively inhibited by ligands of AT₄ receptors, including Ang IV, LVVYPWTQRF (SEQ ID NO:2) and KYLPGPLQ (SEQ ID NO:3). IRAP binds Ang IV with an affinity of 20 to 30 nM. We have demonstrated by cross-linking studies that the AT₄ receptor, like IRAP, is a glycoprotein of molecular size 165 kDa. We were able to cross-link ^{125}I -Nle Ang IV-BP to HEK 293-T cells which had been transfected with IRAP cDNA in an expression plasmid, but not to cells transfected with control plasmids.

We have also demonstrated, using *in situ* hybridization histochemistry with antisense oligonucleotides and by immunohistochemistry using an antibody raised against mouse IRAP, that the distribution of IRAP and its mRNA parallels that of the AT₄ receptor. We have therefore demonstrated that IRAP is the AT₄ receptor.

We have also shown that Nle-Ang IV and LVVYPWTQRF (SEQ ID NO:2) inhibit the aminopeptidase activity of IRAP *in vitro*. We therefore believe that *in vivo* modulation of the enzyme by peptides such as Ang IV, Nle¹-Ang IV, LVVYPWTQRF (SEQ ID NO:2) and KYLPGPLQ (SEQ ID NO:3) activates neurones in the central nervous system to enhance learning and memory. Structure-activity studies performed on these peptides and on known inhibitors of leucine-aminopeptidases will enable us to develop peptidomimetic and other drugs which modify the IRAP enzymatic activity.

This invention unites findings on the effects of peptide and non-peptide ligands of the AT₄ receptor on the central nervous, cardiovascular and renal systems with the effects of

- (a) candidate substrates of IRAP, and
- (b) protein constituents of GLUT4 vesicles.

Both IRAP and GLUT4 are known to translocate to the cell surface in response to insulin in adipocytes, skeletal muscles and cardiac myocytes, which is the predominant glucose-transporting mechanism in these cells.

5 Although the roles of IRAP in these cells are unknown, its close association with GLUT4 is suggestive of a role for the enzyme in the regulation of glucose uptake and its storage.

Numerous conclusions can be drawn from our

10 results. For example, the dramatic effects of AT₄ receptor ligands on memory and learning in normal animals and reversal of amnesia induced by cholinergic antagonists and bilateral perforant pathway lesions may be mediated by

(1) modulation of glucose transport in neurones

15 (2) modulation of extracellular and intracellular concentrations of neuropeptides such as vasopressin, oxytocin, somatostatin, angiotensin III, Lys-bradykinin, neurotensin, met-enkephalin, dynorphin A, neurokinin A, neuromedin B, and cholecystokinin 8, which are all

20 thought to be substrates of the enzyme *in vitro*, and/or

(3) modulation of the levels of polypeptides such as insulin, IGF-I and IGF-II.

The activity of the AT₄ receptor may also be regulated by proteins known to interact with IRAP, such as

25 tankyrase; proteins which are associated with GLUT4 vesicles, such as sortilin and IGF-II receptor; and proteins which regulate GLUT4 vesicle translocation, such as Munc 18c, Syntaxin 4, SNAP23, Synip, and VAMP 2 (Pessin et al, 1999, Cheatham, 2000, Foster and Klip, 2000,

30 Simpson et al, 2001, Holman and Sandoval, 2001).

This invention also relates to novel methods for the development of modulators of AT₄ receptor/IRAP activity. These compounds will lead to an alteration in the levels of

35 (1) a number of neuropeptides, such as arginine-vasopressin, oxytocin, somatostatin, angiotensin III, Lys-

bradykinin, neurotensin, met-enkephalin, dynorphin A, neurokinin A, neuromedin B, cholecystokinin 8, and
(2) a number of polypeptides, such as insulin, insulin-like growth factor-I and insulin-like growth
5 factor-II.

The development of modulators of AT₄ receptor/IRAP activity may for example be based on
(a) modification of peptides which bind to AT₄ receptor/IRAP, such as VYIHPF (SEQ ID NO:1), LVVYPWTQRF
10 (SEQ ID NO:2), and KYLPGPLQ (SEQ ID NO:3);
(b) peptidomimetic compounds based on the peptide structures of known modulators of IRAP;
(c) compounds based on the structure of known inhibitors of the family of leucine aminopeptidases, such
15 as L-leucinethiol, L-leucinal, or L-leucinol; and
(d) compounds which interfere with the interaction of IRAP with other proteins which regulate IRAP activity

The invention provides an assay to screen for compounds which modulate the activity of IRAP, and a
20 diagnostic marker for disorders related to alterations in the activity of IRAP in serum or in tissues.

The modulators of IRAP activity preferably result in a decrease in the catalytic activity of IRAP. These compounds are useful for increasing serum and tissue
25 levels of biologically active agents such as arginine-vasopressin, oxytocin, somatostatin, angiotensin III, Lys-bradykinin, neurotensin, met-enkephalin, dynorphin A, neurokinin A, neuromedin B, cholecystokinin 8, insulin, IGF-I or IGF-II. Therefore administration of modulators
30 of IRAP activity can have the same effects or uses as administration of exogenous arginine-vasopressin, oxytocin, insulin, IGF-I or IGF-II.

Compounds which modulate the activity of IRAP may be used in the treatment, prevention or diagnosis of
35 conditions such as

1. neurodegenerative disorders, including Alzheimer's disease and other forms of dementia;

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2. other disorders of the CNS, particularly those involving motor and sensory systems;
3. disorders of the CNS resulting from trauma or stroke;
- 5 4. disorders of the cardiovascular system, including cardiac hypertrophy, congestive heart failure, hypertension, and atherosclerosis;
5. disorders of development and/or growth;
6. disorders of the reproductive tract;
- 10 7. disorders associated with pregnancy;
8. disorders of glucose and fat metabolism, including syndrome X, hyperlipidemia, and diabetes;
9. cancer; or
10. premature or delayed labour.
- 15 Such compounds may also be used to accelerate wound healing.

Unless otherwise indicated, the following examples use conventional molecular biology, cellular biology, and recombinant DNA techniques. Such techniques

20 are well known to the skilled worker, and are explained fully in the literature. See, for example, Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual" (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover, ed., 1985);

25 "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Nucleic Acid Hybridisation" (B.D. Hames & S.J. Higgins, eds., 1985); "Transcription and Translation" (B.D. Hames & S.J. Higgins, eds., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1986); "Immobilised Cells and Enzymes" (IRL

30 Press, 1986); B. Perbal, "A Practical Guide to Molecular Cloning" (1984), and Sambrook, et al., "Molecular Cloning: a Laboratory Manual" (1989). Ausubel, F. et al., 1989-1999, "Current Protocols in molecular Biology" (Green Publishing, New York).

35 In describing the present invention, the following terminology is used, in accordance with definitions set out below.

The terms "AT₄ receptor", "AT₄ receptor/IRAP" and "IRAP" are used interchangeably. While hitherto they have been regarded as being separate molecules, we have now determined that they are one and the same.

5 The terms "nucleic acid" and "polynucleic acid" refer herein to deoxyribonucleic acid and ribonucleic acid in all their forms, i.e., single and double-stranded DNA, cDNA, mRNA, and the like.

10 The terms "antisense" oligonucleotides generally refers to small, synthetic oligonucleotides resembling single-stranded DNA, which are applied to the inhibition of gene expression by inhibition of a target messenger RNA (mRNA). See Milligan, J.F. et al., J.Med.Chem 36 (14), 1923 - 1937 (1993), the relevant portion of which is
15 hereby incorporated by reference. These oligonucleotides inhibit gene expression of target genes. This is generally attained by hybridisation of the antisense oligonucleotides to coding (sense) of a targeted mRNA, as is known in the art. The exogenously administered
20 antisense agents of the invention decrease the levels of mRNA and protein encoded by the target gene, and/or cause changes in the growth characteristics or shapes of thus treated cells. See Milligan et al., (1993); Helene, C. and Toulme, J. Biochim. Biophys. Acta 1049, 99-125 (1990);
25 Cohen, J. S. D. Ed., Oligonucleotides as antisense inhibitors of gene expression; CRC Press: Boca Raton, F L (1987), the relevant portions of which are hereby incorporated by reference. As used herein, "antisense oligonucleotide" is generally a short sequence of
30 synthetic nucleotide which hybridises to any segment of a mRNA encoding a targeted protein under appropriate hybridisation conditions, and which upon hybridisation causes a decrease in expression of the targeted protein.

 As used herein, the term "treat" or "treating"
35 refers to a treatment which decreases or abolishes the likelihood that a subject administered such treatment will manifest symptoms of an AT₄ receptor/IRAP-related disease.

As used herein, "functionally active" and "functional activity" in reference to the peptides of the invention means that the compounds generated therefore are able to modulate the activity of insulin-regulated aminopeptidase (IRAP). The activities of IRAP include

- (a) its aminopeptidase activity
- (b) AT_4 receptor activity
- (c) signalling activity such as activation of second messenger pathways, and
- (d) modulating glucose transport.

Therefore, a functionally active compound of the present invention is capable of competitively inhibiting, stimulating or modulating one or more of these activities.

Amino acid sequence variants of the peptides of the invention are prepared by introducing appropriate amino changes into amino acid sequence, or by *in vitro* synthesis. Such variants include, for example, deletions from, or insertions or substitutions of, amino acid residues within the peptide sequences disclosed. Any combination of deletion, insertion, and substitution may be made to arrive at an amino acid sequence variant of the peptides of the invention, provided that such a variant possesses the desired characteristics described herein.

Covalent modifications of the peptides of the invention are also included within the scope of this invention. For example, covalent modifications may be introduced into the peptides of the invention by reacting targeted amino acid residues with an organic derivatizing agent which is capable of reacting with selected amino acid side chains or the N- or C-terminal residues.

Cysteinyll residues most commonly are reacted with α -haloacetates and corresponding amines, such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyll residues also are derivatized by reaction with bromotrifluoroacetone, α -bromo- β -(5-imidozoyl)propionic acid, chloroacetyl phosphate N-alkylmaleimides, 3-nitro-2-

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pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction
5 with diethyl-pyro-carbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0. Lysinyl and amino terminal residues are reacted with
10 succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α -amino-containing residues include imidoesters such as methyl
15 picolinimide; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4-pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with
20 one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group.
25 Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic
30 diazonium compounds or tetranitromethane. Most commonly, N-acetylimidazole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl side groups such as aspartyl or glutamyl
35 are selectively modified by reaction with carbodiimides ($R'-N=C=N-R'$), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)

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carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

5 Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues respectively. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of
10 this invention.

 Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α -amino groups of lysine, arginine, and histidine side chains,
15 acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. See Creighton, Proteins: Structure and Molecular Properties, pp.79-86 (W.H. Freeman & Co., 1983).

 The term "more resistant to metabolic
20 degradation" means that the compound of the invention has been modified such that the resulting compound is more stable under acidic conditions than the "native" sequence of the peptides of the invention. For example, amino acid substitutions as discussed previously may be undertaken
25 which produce compounds more resistant to metabolic degradation. It is well known in the art that D-amino acids, and amino acid analogues are more resistant to acidic environments. Conjugates of small peptides and cholic acid have reduced metabolic degradation problems.

30 The term "biological activity" with reference to AT₄ receptor/IRAP means a biological activity which is normally promoted, either directly or indirectly, by the presence of AT₄ receptor/IRAP, and includes, but is not limited to, AT₄ receptor/IRAP binding and biochemical
35 effects such as modulation of the enzymatic, signalling, and glucose transport activities of IRAP, and modulation of the AT₄ receptor activities, including enhancement of

memory, reversal of amnesia and growth promotion. Such biological activities can be measured by conventional *in vitro* and *in vivo* assays, including enzymatic assays using a synthetic substrate, peptide degradation assays using HPLC, behavioural assays for memory and learning such as the Barnes maze, and glucose uptake assays in cells, synaptosomes and whole animals.

It will be clearly understood that the compounds of the invention may be synthesised by any suitable method. Solid phase methods, such as those developed for synthesis of peptides and peptidomimetic compound, are preferred, including but not limited to the Fmoc solid phase peptide synthesis method described herein, the Boc solid phase peptide synthesis method, and pin synthesis methods (for review, see Maeji et al., 1995). Those skilled in the art will readily be able to select the most suitable method for any given compound of the invention.

In one preferred embodiment of this invention, the phosphodiester residues of the antisense oligonucleotide are modified or substituted. Chemical analogues of oligonucleotides with modified or substituted phosphodiester residues, eg, to the methylphosphonate, which increase the *in vivo* stability of oligonucleotide are particularly preferred. The naturally occurring phosphodiester linkages of oligonucleotides are susceptible to some degree of degradation by cellular nucleases. Many of the residues proposed herein, on the contrary, are highly resistant to nuclease degradation. See Milligan et al, and Cohen, J. S D., Supra. In another preferred embodiment of the invention the oligonucleotides may be protected from degradation by adding a "3'-end cap", by which nuclease-resistant linkages are substituted for phosphodiester linkages at the 3' end of the oligonucleotide. See Tidd D. M. and Warenrus, H. M., Be. J. Cancer 60:343-350 (1989); Shaw, J.P. et al., Nucleic acids research. 19:747-750 (1991), the relevant sections of which are incorporated herein by reference.

Phosphoroamidates, phosphorothioates, and methylphosphonate linkages all function adequately in this manner for the purposes of this invention. The more extensive the modification of the phosphodiester backbone, the more stable the resulting agent, and in many instances the higher their RNA affinity and cellular permeation. See, Milligan et al., supra. Thus the number of residues which may be modified or substituted will vary, depending on the need, target and route of administration, and may be from one to all residues, or any number in between. Many different methods for replacing the entire phosphodiester backbone with novel linkages are known. See Milligan et al., Supra. Preferred backbone analogue residues include phosphorothioate, formacetal boranophosphate, 3'-thioformacetal, 5'-thioether, carbonate, 5'-N-carbamate, sulphate, sulfonate, sulfamate, sulfonamide, sulfone, sulfite, 2'-O methyl, sulfoxide, sulfide, hydroxylamine, methylene (methylimino) (MMI), methyleneoxy (methylimino) (MOMI) residues.

Phosphorothioate and methylphosphate-modified oligonucleotides are particularly preferred, due to their availability through automated oligonucleotide synthesis. See Milligan et al., Supra. Where appropriate the agents of this invention may be administered in the form of their pharmaceutically acceptable salts, or as a mixture of the antisense oligonucleotide and its salt. In another embodiment of this invention, a mixture of different antisense oligonucleotides or their pharmaceutically acceptable salts is administered.

The antisense nucleic acid molecules (agents) of this invention may be of any suitable length, for example about 7 to about 60 nucleotides long, preferably about 12 to about 45, more preferably up to about 30 nucleotides long, and still more preferably up to about 21, although they may be other lengths as well, depending on the particular target and the mode of delivery. The agents of the invention may be directed to any and all segments of a

target RNA.

In a further embodiment of the present invention, the oligonucleotide is operatively linked to an agent or molecule which is itself internalised or taken up by
5 living cells. In this manner, the uptake of the agent of the invention is enhanced. Examples of agents or molecules suitable for use with the oligonucleotides of this invention are transferrin asialoglycoprotein and streptavidin. Other agents will be known to those skilled
10 in the art.

The invention also provides a pharmaceutical composition comprising an antisense oligonucleotide in an amount effective to reduce expression of a target mRNA, by passing through a cell membrane and binding specifically
15 with target mRNA in the cells, so as to prevent its translation, together with a suitable pharmaceutically acceptable carrier, eg. sterile pyrogen-free saline solution. The agent of the invention may be formulated with a hydrophobic carrier capable of passing through a cell membrane, eg in a liposome, with the liposomes
20 carried in a pharmaceutically acceptable aqueous carrier. The oligonucleotides may be coupled to an agent which inactivates mRNA, such as a ribozyme. Such oligonucleotides may be administered to a subject in need
25 of such treatment to inhibit the activation of specific receptors, enzymes and/or proteins and/or factors. The pharmaceutical formulation may also comprise chimeric molecules comprising antisense oligonucleotides attached to molecules which are known to be internalised by cells.
30 These oligonucleotide conjugates utilise cellular uptake pathways to increase intercellular concentrations of the oligonucleotide. Examples of molecules used in this manner include macromolecules, including transferrin, asialoglycoprotein (bound to oligonucleotides via
35 polylysine) and streptavidin. The antisense compound may be contained in the pharmaceutical formulations within a lipid particle or vesical, such as a liposome or

microcrystal. The particles may be of any suitable structure, such as unilamellar or plurilamellar. In one preferred embodiment, the antisense oligonucleotide is comprised within the liposome. Positively charged lipids
5 such as N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammoniummethylsulfate, or "DOTAP", are particularly preferred for such particles and vesicles. However, others are also suitable. The preparation of such lipid particles is well-known. See, for example, US
10 Patent Nos. 4,880,635 to Janoff et al, 4,906,477 to Kurono et al., 4,911,928 to Wallach, 4,917,951 to Wallach, 4,920,016 to Allen et al., 4,921,757 to Wheatley et al., the relevant sections of all of which are herein incorporated in their entireties by reference.

15 The pharmaceutical compositions provided herein may comprise the antisense oligonucleotides described above and one or more surfactants. Suitable surfactant components for enhancing the up-take of the antisense oligonucleotides of the invention include synthetic and
20 natural as well as full length and truncated forms of surfactant protein A, surfactant protein B, surfactant protein C, surfactant protein D and surfactant protein E, di-saturated phosphatidylcholine (other than dipalmitoyl, dipalmitoyl phatidylcholine, phosphatidylcholine,
25 phosphatidylglycerol, phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine; phosphatidic acid, ubiquinones, lysophosphatidylethanolamine, lysophosphatidylcholine, palmitoyl-lysophosphatidylcholine, dehydroepiandrosterone,
30 dolichols, sulfatidic acid, glycerol-3-phosphate, dihydroxyacetone phosphate, glycerol, glycerol-3-phosphocholine, dihydroxyacetone, palmitate, cytidine diphosphate (CDP) diacylglycerol, CDP-choline, choline, choline phosphate; as well as natural and artificial
35 lamellar bodies which are the natural carrier vehicles for the components of surfactant, omega-3 fatty acids, polyenic acid, polyenoic acid, lecithin, palmitinic acid,

non-ionic block copolymers of ethylene or propylene oxides, polyoxypropylene, monomeric and polymeric, polyoxyethylene, monomeric and polymeric, poly (vinyl amine) with dextran and/or alkanoyl side chains, Brij 35, Triton X-100 and synthetic surfactants ALEC, Exosurf, Survan and Atovaquone, among others. These surfactants may be used either alone or as part of a multiple component surfactant in a formulation, or as covalently bound additions to the 5' and/or 3' ends of the antisense oligonucleotides (oligos).

As already indicated, the agent of this invention is also provided as a composition, comprising the agent and a carrier. The carrier is preferably a biologically acceptable carrier, and more preferably a pharmaceutically or veterinarily acceptable carrier in the form of gaseous, liquid, or solid carriers, and mixtures thereof, which are suitable for the different routes of administration intended. The composition may optionally comprise other agents such as other therapeutic compounds known in the art for treatment of the condition or disease, antioxidants, flavouring and colouring agents, fillers, volatile oils, buffering agents, dispersants, surfactants, RNA-inactivating agents, antioxidants, flavouring agents, propellants and preservatives, as well as other agents known to be utilised in therapeutic compositions. An example of the mRNA inactivating agent is an enzyme, such as ribozyme.

The composition generally contains the antisense oligonucleotide in an amount of about 0.01 to about 99.99 w/w, preferably about 1 to about 40 w/w, and more preferably about 5 to about 20 w/w of the composition. However, other ingredients, and other amounts of the agent are also suitable within the confines of this invention.

The agent of the invention is also provided in various formulations which are tailored for different methods of administration and routes of delivery. The formulations contemplated are, for example, a transdermal

formulation also containing carrier(s) and other agents suitable for delivery through the skin, mouth, nose, vagina, anus, eyes, ears, other body cavities, intradermally, as a sustained release formulation, intracranial, intrathecally, intravascularly, by inhalation, intrapulmonarily, into an organ, by implantation, including suppositories, creams, gels, and the like, as is known in the art. In one particular formulation, the agent is suspended or dissolved in a solvent. In another the carrier comprises a hydrophobic carrier, such as lipid particles or vesicles, including liposomes and micro crystals. The preparation of all of these formulations, as well as the ingredients to be utilised, are known in the art, and need not be further described here. In one particularly preferred embodiment of the vesicle formulation, the vesicles comprise liposomes containing the antisense oligonucleotides. The lipid vesicles may comprise N-(1-[2,3-dioleoxylpropyl)-N,N,N-trimethyl-ammonium methylsulfate as well as other lipids known in the art to provide suitable delivery of DNA to target cells. In one embodiment, the formulation comprises a respirable formulation, such as an aerosol. The agent, composition, and formulation of the invention are provided in bulk, and in unit form, as well as in the form of an implant, a solution or suspension, a capsule or cartridge, which may be openable or piercable as is known in the art. A kit is also provided, which comprises a delivery device, and in separate containers, the agent, composition or formulation of the invention, and optionally other agents, and instructions for the use of the kit components.

The kit may optionally also comprise in a separate container an agent selected from the group consisting of other therapeutic compounds, antioxidants, flavouring and colouring agents, fillers, volatile oils, buffering agents, dispersants, surfactants, cell internalisation or up-take agents, RNA-inactivating

agents, antioxidants, flavouring agents, propellants and preservatives, among other suitable additives for the different formulations. When a solvent for the agent or other ingredients is added, organic solvents and organic solvents mixed with one or more co-solvents may be utilised as well as aqueous solvents as is known in the art.

The agent of the invention may be provided in conjunction with a vector for delivery purposes, or for manufacturing copies thereof. The agent may be operatively linked to the vector, using methods known in the art. The agent may also be provided within a host cell for amplification of the oligonucleotide, and for storage purposes.

EXAMPLE 1 PURIFICATION AND IDENTIFICATION OF THE
LIGAND-BINDING SUBUNIT OF THE AT₄ RECEPTOR
AND INTERACTING PROTEINS

Since the DNA sequences encoding the AT₄ receptor had not been previously identified, we wished to purify the receptor protein and obtain peptide sequence by mass spectrometry, which would allow us to identify the gene encoding the receptor. The purification procedure is described below.

Bovine adrenals were homogenised with a polytron homogeniser in hypotonic buffer (50mM Tris-HCl pH 7.4, 5mM EDTA, pH 7.4). Tissue debris was removed by centrifugation at 600g for 5min at 4°C. The supernatant was centrifuged at 50,000g for 20min at 20°C and the membrane pellet was resuspended in isotonic buffer consisting of 50mM Tris-HCl pH 7.4, 150mM NaCl, 5mM EDTA, 20µM bestatin, 100µM PMSF, 0.1% BSA.

To covalently cross-link [¹²⁵I]-Nle¹-BzPhe⁶-Gly⁷-Ang IV-benzoylphenylalanine ([¹²⁵I]-Nle-Ang-IV-BP) to the receptor, bovine adrenal membranes (1.6µg of protein) were incubated with 0.1µCi [¹²⁵I]-Nle-Ang-IV-BP in 20ml of isotonic buffer in Eppendorf tubes (1ml per tube) at 37°C

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with shaking for 90min in the dark. Membranes were collected by centrifugation at 60,000g for 10min at 4°C, and resuspended in 500µl of ice cold isotonic buffer and UV irradiated at 365nm on ice for 2h. Membranes were again
5 collected by centrifugation at 60,000g for 20 min at 4°C.

To solubilise the receptor, cross-linked membranes were homogenised in 2ml solubilisation buffer (1% CHAPS, 20mM Tris-HCl, pH 7.5, 5mM EDTA) in a Dounce homogeniser, and shaken at room temperature for 48h.

10 Insoluble material was pelleted by centrifugation at 100,000g for 1h at 4°C. Non-crosslinked membranes (48mg of protein) were solubilised and centrifuged similarly, and the supernatant combined with that from cross-linked membranes.

15 To enrich for the receptor, solubilised membrane protein was applied to a 1ml DEAE fast flow anion exchange column. The column was then washed with 5ml of solubilisation buffer, then with 5ml of solubilisation buffer plus 50mM NaCl. AT₄ receptor was eluted with
20 solubilisation buffer plus 150mM NaCl and collected in 0.5ml fractions. Proteins in 200µl aliquots of eluted fractions were resolved by 7.5% SDS-PAGE and the gel stained first with Coomassie brilliant blue and then overstained with silver nitrate (Shevchenko et al. 1996).
25 Radioactive bands were imaged after exposing to phosphorimager plates for 16h, as illustrated in Figure 1.

The silver-stained band that co-migrated with the major radioactive band at 165 kDa was analysed by mass spectrometry as follows.

30 The protein band was excised, reduced, alkylated and digested with trypsin *in situ* (Moritz et al., 1996). Tryptic peptides were subjected to capillary-column reversed phase-high performance liquid chromatography (RP-HPLC; Hewlett Packard Model 1090A modified for capillary
35 chromatography), coupled on-line with an electrospray ionization (ESI) ion trap (IT) mass spectrometer (LCQ Finnigan MAT, San Jose, CA, USA) for peptide sequencing.

The column used was a 150 x 0.20mm ID capillary column (Brownlee RP-300, 7 μ m C8) manufactured using a PVDF end frit. A 60 min linear gradient (flow rate 1.7 μ L/min) was used from 0-100% B, where solvent A was 0.1% v/v aqueous TFA and solvent B was 0.1% v/v aqueous TFA in 60% acetonitrile. The ESI parameters were as follows: spray voltage, 4.5kV; sheath gas and auxiliary gas flow rates, 5 and 30 (arbitrary value), respectively; capillary temperature, 150°C; capillary voltage, 20V; and tube lens offset 16V. The sheath liquid used was 2-methoxyethanol (99.9% HPLC grade) delivered at a flow rate of 3 μ L/min. The electron multiplier was set to -860 V. In both MS and MS/MS modes, the trap was allowed a maximum injection time of up to 200ms. The automatic gain control parameter was turned on for all experiments, ensuring that the number of ions in the trap was automatically kept to a constant preset value. The range scanned in MS mode was 350-2000 Da, and in MS/MS the range varied according to the mass of the ion selected for MS/MS. After acquiring one scan in MS, the most intense ion in that spectrum above a threshold of 1×10^5 was isolated for subsequent zoom scan to determine charge state, then collision-induced dissociation or MS/MS in the following scan. The dissociation energy for MS/MS was set to 55%. All spectra were recorded in centroid mode.

Two MS/MS spectra were identified using the SEQUEST algorithm (Eng et al, 1994; incorporated into the Finnigan Xcalibur-Biomass software) and the Ludwig Institute non-redundant database (Ludwig Institute for Cancer Research, Lausanne branch). These peptides were identified as residues 208-220 and 321-349 of CD34 from *Bos taurus* (TrEMBL accession number Q9XS61), as illustrated in Figure 2. One MS/MS spectrum not identified by SEQUEST was deduced by manual amino acid sequence interpretation to be AHLSEVQAFFENQSEATFR (SEQ ID NO:5). A search of the database revealed 18 of the 19 amino acids to be identical to residues 979-996 of human

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oxytocinase (TrEMBL accession number 000769), the human
homologue of rat insulin-regulated aminopeptidase (IRAP)
(Figure 2). The derived bovine sequence has an alanine
residue at the N-terminus, whereas the human sequence has
5 a threonine.

Further enrichment of the AT₄ receptor was
achieved by coupling anion exchange chromatography to
wheat germ lectin affinity chromatography, as described in
Example 3.

10

EXAMPLE 2 CONFIRMATION THAT THE AT₄ RECEPTOR IS IRAP

To confirm that the AT₄ receptor is IRAP, we
obtained plasmids containing the human IRAP cDNA (Rogi et
al, 1996) from Dr Masafumi Tsujimoto, and transfected them
15 into HEK 293 cells. Membranes prepared from these cells
were incubated with ¹²⁵I-Nle Ang IV in the absence and
presence of increasing concentrations of Ang IV (Figure 3)
LVVYPWTQRF (SEQ ID NO:2) (Figure 4) and KYLPGPLQ (SEQ ID
NO:3) (Figure 5) respectively. The amount of radioligand
20 bound to the membranes from HEK 293 cells transfected with
IRAP cDNA was expressed as a percentage of radioligand
bound to membranes from HEK 293 cells transfected with
control plasmids. Cross-linking experiments were also
carried out on the membranes from HEK 293 cells
25 transfected with the IRAP cDNA, using the method described
in Example 1 (Figure 6).

Membranes from HEK 293 cells transfected with
IRAP cDNA produced a 25-35 fold increase in the total
number of binding sites, compared to membranes prepared
30 from cells transfected with control plasmids. The
affinities of Ang IV and LVVYPWTQRF (SEQ ID NO:2) in
competing for the binding of ¹²⁵I-Nle Ang IV in the IRAP-
transfected cells were not significantly different from
that of the endogenously-occurring IRAP in HEK 293 cells
35 (Figures 3 and 4). The cross-linking experiment revealed
that at least two radiolabelled bands were present on the
SDS-PAGE gel, a major band of approximately 165 kDa and a

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minor band of greater than 250 kDa (Figure 6). These results demonstrated a dramatic increase in AT₄ receptor in the HEK 293 cells transfected with the IRAP cDNA, and confirmed that IRAP is the AT₄ receptor.

5 We also investigated the distribution of IRAP, using an antibody raised in rabbits against a GST fusion protein containing the cytoplasmic tail of murine IRAP, and the distribution of IRAP mRNA, using *in situ* hybridization with antisense oligonucleotides generated
10 from the mouse IRAP cDNA sequence. The antisense sequences used include
5'GAGGTTAGGTTTGGATGTAGGCTAAGTTCAT 3' (SEQ ID NO:6) and
5'ATTGATCAGGTTGGCTCGGTCTTTGTCA 3' (SEQ ID NO:7)
from the mouse IRAP cDNA sequence retrieved from the
15 CELERA database. The distribution of IRAP-positive immunoreactivity and IRAP mRNA closely correlated with the distribution of AT₄ receptor, as determined using ¹²⁵I-Nle Ang IV binding in mouse brain (Figure 7) and embryo (Figure 8). The distribution of IRAP mRNA in human
20 cerebellum was demonstrated by *in situ* hybridization histochemistry, using antisense and sense oligonucleotide sequences derived from the human IRAP cDNA sequence. The antisense sequences used include nucleotides 1136-1109, nucleotides 1407-1377 and nucleotides 2587-2560 of the
25 human IRAP sequence (Human Genbank Acc.U62768). The adjacent sections of the cerebellum were labelled with ¹²⁵I-Nle Ang IV to demonstrate AT₄ receptor binding. The results are shown in Figure 9.

30 EXAMPLE 3 DIFFERENT ISOFORMS OF AT₄ RECEPTOR/IRAP

The affinity of AT₄ receptor/IRAP for its ligands varies considerably between tissues and cell types. This may be due to interactions of the ligand binding subunit with other proteins which modulate the binding affinity.
35 Alternatively, different isoforms of the enzyme may exist. In support of the latter conclusion, an IRAP-related EST (Genbank accession #BF381719) which includes intron 2

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sequence following exon 1, was identified from a library made from a cell line derived from human primitive neuroectoderm. This could represent a tissue- or stage-specific variant of IRAP arising by alternative splicing.

5 The existence of brain-specific variant of AT₄ receptor/IRAP may make it possible to develop modulators of AT₄ receptor/IRAP which specifically target the brain form of the receptor, and which therefore do not have unwanted side-effects in peripheral tissues.

10

EXAMPLE 4 IDENTIFICATION OF PROTEINS WHICH INTERACT WITH THE LIGAND-BINDING SUBUNIT OF IRAP/AT₄ RECEPTOR BY SDS-PAGE GELS AND IDENTIFICATION BY MASS SPECTROMETRY

15 When AT₄ receptor/IRAP from bovine adrenal membranes was cross-linked to [¹²⁵I]-Nle-Ang IV-BP and solubilised in 1% NP40 or 1% CHAPS and the proteins resolved by SDS-PAGE, one, two or three minor radiolabelled higher molecular weight complexes were

20 observed (Figure 10). These migrated with apparent molecular weights ranging from approximately 180 to 300kDa. These results suggested that the ligand-binding subunit of AT₄ receptor/IRAP existed as a multi-subunit complex. A 250kDa complex was also observed when the AT₄ receptor/IRAP from SK-N-MC cells was cross-linked to

25 [¹²⁵I]-Nle-Ang IV-BP and the proteins resolved by SDS-PAGE under non-reducing conditions, but not under reducing conditions (Figure 11), suggesting that a subunit interacts with the ligand-binding subunit through

30 disulphide bonds.

In order to determine the mechanism by which AT₄ receptor/IRAP influences other physiological events, the identification of proteins which complex with the ligand binding subunit of IRAP/AT₄ is required. We hypothesise

35 that the interactions may modulate the function of AT₄ receptor/IRAP by inhibiting or enhancing its catalytic activity. Alternatively, the binding of peptides to AT₄

receptor/IRAP may activate a second messenger system via proteins which interact with IRAP. AT₄ receptor/IRAP-interacting proteins might also have roles in the intracellular trafficking of AT₄ receptor/IRAP. Molecules which interact with the AT₄ receptor/IRAP represent potential targets for drugs designed to reverse memory deficits in conditions such as Alzheimer's disease and other forms of dementia, or to modulate other physiological actions of the IRAP/AT₄ system. If an interacting molecule shows a more restricted distribution than does AT₄ receptor/IRAP (e.g. limited to brain or certain regions of the brain), it may provide a more suitable target than is IRAP for a drug which specifically modulates the effects of the IRAP/AT₄ system in a particular tissue or cell type.

To achieve further enrichment for the AT₄ receptor/IRAP, with a view to isolating the higher molecular weight complexes, ion exchange purification was scaled up and fractions enriched for the receptor were further purified by wheat germ lectin affinity chromatography. The two-step purification was carried out as follows: [¹²⁵I]-Nle-Ang IV-BP was cross-linked to the AT₄ receptor in bovine adrenal membranes (93mg protein) and solubilised as described in Example 1. Non-crosslinked receptor from bovine adrenal membranes (254mg of protein) was solubilised and combined with cross-linked receptor. The receptor was enriched by DEAE-Sepharose fast flow anion exchange as described above. Fractions enriched for the receptor were then applied to a 1ml wheat-germ lectin-agarose column, the column was washed with 5ml of solubilisation buffer, and the receptor eluted with 0.5M N-acetylglucosamine, 0.5M NaCl in solubilisation buffer, and collected in 0.5ml aliquots. Proteins in 200μl aliquots of eluted fractions were resolved by 7.5% SDS-PAGE, and the gel stained and autoradiographed as described above. The ligand-binding subunit was visualised by Coomassie blue staining, and bands which co-

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migrated with two of the higher molecular weight complexes were barely visible after silver staining. These bands were excised from the gel and analysed by capillary-column RP-HPLC coupled with ESI-IT-MS/MS, as described above.

- 5 The resulting CID spectra of the peptides were compared with those of proteins in the database.

To identify proteins interacting with AT₄ receptor/IRAP in a tissue of interest, AT₄ receptor/IRAP from that tissue was cross-linked to [¹²⁵I]-Nle-Ang IV-BP, 10 solubilised and purified by ion exchange chromatography and wheat germ lectin affinity chromatography, as described above, and proteins resolved by SDS-PAGE. Silver or Coomassie blue-stained bands which co-migrated with radiolabelled bands corresponding to higher molecular 15 weight complexes were excised from the gel and analysed by capillary-column RP-HPLC coupled with ESI-IT-MS/MS, as described above.

Two bands corresponding to 250 and 300 kDa were excised from the SDS-PAGE gel, and subjected to alkylation 20 and digestion with trypsin as described above. Tryptic peptides were subjected to capillary-column reversed phase-high performance liquid chromatography, coupled on-line with an electrospray ionization (ESI) ion trap (IT) mass spectrometer for peptide sequencing as described 25 above.

For the 250 kDa gel band,

1. Twenty-five MS/MS spectra were identified, and the peptides correspond to internal sequences of voltage-dependent calcium channel alpha-2 delta subunit precursor 30 - *Sus scrofa* (TrEMBL accession number 077773).
2. Four MS/MS spectra were identified, and the peptides correspond to internal sequences of integrin beta-1 (fibronectin receptor beta subunit) - *Bos taurus* (SwissProt P53712).
- 35 3. Two MS/MS spectra were identified, and the peptides correspond to internal sequences of cytochrome

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p450 11A1, mitochondrial precursor (cholesterol side-chain cleavage enzyme) - *Bos taurus* (SwissProt P00189)

4. One MS/MS spectrum was identified, and the peptide corresponded to an internal sequence of receptor Dec205

5 (Ly75) - *Mus musculus* (TrEMBL Q60767)

5. One MS/MS spectrum was identified, and the peptide corresponded to an internal sequence of dopamine beta-monooxygenase precursor - *Bos taurus* (SwissProt P15101).

10 For the 300 kDa gel band,

1. Five MS/MS spectra were identified, and the peptides correspond to internal sequences of cation-independent mannose-6-phosphate receptor precursor - *Bos taurus* (SwissProt P08169)

15 2. Five MS/MS spectra were identified, and the peptides correspond to internal sequences of voltage-dependent calcium channel alpha-2 delta subunit precursor - *Sus scrofa* (TrEMBL accession number 077773).

3. Four MS/MS spectra were identified, and the
20 peptides correspond to internal sequences of cytochrome p450 11A1, mitochondrial precursor (cholesterol side-chain cleavage enzyme) - *Bos taurus* (SwissProt P00189)

4. One MS/MS spectrum was identified, and the peptide corresponded to an internal sequence of dopamine
25 beta-monooxygenase precursor - *Bos taurus* (SwissProt P15101).

EXAMPLE 5 ISOLATION AND IDENTIFICATION OF INTERACTING
PROTEINS BY CO-IMMUNO-PRECIIPITATION OR
IMMUNOAFFINITY CHROMATOGRAPHY USING
5 ANTIBODIES AGAINST IRAP/AT₄ RECEPTOR

To identify proteins interacting with AT₄ receptor/IRAP in tissues of interest, membranes are prepared from these tissues and membrane proteins solubilised with NP40 or CHAPS as described in Example 1,
10 and incubated with antibodies against IRAP and protein G-Sepharose beads. As a control, solubilised membranes are incubated with protein G-Sepharose alone. Precipitated proteins are resolved by SDS-PAGE and detected by Coomassie blue or silver staining. Bands appearing in the
15 test but not in the control lanes are excised and digested with trypsin *in situ*, and tryptic peptides analysed by capillary-column RP-HPLC coupled with ESI-IT-MS/MS, as described in example 1. In a similar approach, proteins complexing with IRAP/AT₄ are isolated by immunoaffinity
20 chromatography using IRAP antibody coupled to a chromatography resin, and identified by tandem MS as described above.

Tankyrase, a Golgi-associated MAP kinase substrate has been shown to interact with IRAP in
25 adipocytes (Chi and Lodish, 2000). Other potential IRAP-interacting proteins include GLUT4 and various proteins involved in GLUT4 trafficking including Syntaxin 4, SNAP23, Synip, Munc18c and VAMP2 (Pessin et al, 1999; Cheatham, 2000; Foster and Klip, 2000; Simpson et al,
30 2001; Holman and Sandoval, 2001). Whether IRAP complexes with these proteins in brain or other tissues or cells of interest is assessed by blotting SDS-PAGE gels containing proteins purified by immunoaffinity chromatography or immunoprecipitation with IRAP antibodies, as described
35 above, and probing with antibodies against the candidate interacting proteins.

EXAMPLE 6 USE OF THE YEAST TWO-HYBRID SYSTEM TO
IDENTIFY FACTORS THAT INTERACT WITH IRAP/ AT₄
RECEPTOR

Yeast cells are transformed with a vector which
5 expresses the IRAP/AT₄ intracellular or extracellular
domain (the bait) as a fusion with a DNA-binding domain,
such as LexA, and a cDNA library in a yeast expression
vector that directs expression of cDNAs fused to a nuclear
localisation signal and a transcriptional activation
10 domain. Cells expressing cDNAs which interact with the
bait protein are then selected, based on expression of one
or more reporter genes, under the control of the LexA
operator. Candidates which activate transcription
specifically in the presence of the bait-DNA binding
15 domain hybrid are investigated further.

The biological relevance of interactions
identified in the yeast two-hybrid system is assessed by
examining whether the interacting protein is co-expressed
with AT₄ receptor/IRAP in relevant tissues and cell types
20 and by determining whether the interacting protein is co-
immunoprecipitated from relevant tissues or cultured cells
by antibodies against IRAP/AT₄.

EXAMPLE 7 IDENTIFICATION OF ENDOGENOUS PEPTIDES WHICH
25 MODULATE THE ACTIVITY OF IRAP/AT₄ RECEPTOR

We searched for endogenous peptides in the
central nervous system which modulated the activity of AT₄
receptor/IRAP. Conventional peptide purification
procedures in combination with the AT₄ radioreceptor assay
30 were used to monitor the receptor binding activity of an
extract of sheep brain.

The AT₄ radioreceptor assay was used to screen
the sheep brain extracts for AT₄ receptor modulatory
activity. The membranes were prepared using bovine
35 adrenal glands obtained from the abattoir. The adrenals
were diced, homogenized in ice-cold hypotonic buffer (50mM
Tris, 5mM EDTA, pH 7.4) and centrifuged for 10min at 500g.

The supernatants were heated to 60°C for 15min, and centrifuged for 20min at 40,000g. The resulting pellets were re-homogenized in hypotonic buffer. Binding assay mixtures comprised 56µg of adrenal membranes, 0.14µCi of [125I] Ang IV (approximately 260pM), and 10µl of column eluent in a total volume of 270µl in 50mM Tris buffer, pH 7.4, containing 150mM sodium chloride, 5mM EDTA, 100µM phenylmethyl-sulfonyl fluoride, 20µM bestatin, and 0.1% (w/v) bovine serum albumin. The samples were incubated for 2h at 37°C, and the bound ligand was separated on Whatman GF/B glass fibre filters. The relative potency of the fractions in competing for 125I-Ang IV binding was determined from a standard curve, in which known amounts of the unlabelled Ang IV were added in concentrations ranging from 10⁻¹⁰ to 10⁻⁶ M.

The same purification procedure was carried out on sheep cerebral cortex which contained blood, and also on blood-free sheep cerebral cortex, in which the sheep brain was perfused with 10 l of ice-cold phosphate-buffered saline solution. The brain tissues were homogenized in 2M acetic acid (2ml/g tissue), centrifuged and the supernatant decanted. A preliminary purification was carried out on the brain extracts using a preparative C18 column (55-105mm, Waters). The C18 eluant was lyophilized, reconstituted and subjected to a series of chromatographic steps in which the fractions with the highest AT₄ receptor modulatory activity were carried through to the next purification step. In brief, three successive reverse-phase HPLC steps involving columns of varying pore size were used (Deltapak C18, and Novapak C18), followed by anion exchange then cation exchange, with a final purification on a microbore LC C8 column. The purified active peptides were then sequenced on an Applied Biosystems Model 470 A Protein Sequencer with an on-line Nodel 120A PTH Analyzer.

A decapeptide having the sequence LVVYPWTQRF (SEQ ID NO:2) was purified from the sheep cerebral cortex which

contained blood. A search of the database revealed that this sequence was present in the internal sequence of β , δ , γ and ϵ globin, and is known as LVV-hemorphin 7. This decapeptide may have resulted from the breakdown of haemoglobin. The purification procedure was repeated on brain which had been perfused to remove all traces of blood, and an octapeptide of sequence KYLPGPLQ (SEQ ID NO:3) was purified. This peptide is a fragment of complexin II, a cytosolic protein located in nerve terminals which is associated with the docking/fusion complex which regulates neurotransmitter release.

Synthetic LVVYPWTQRF (SEQ ID NO:2) and KYLPGPLQ (SEQ ID NO:3) displayed comparable affinities to Ang IV in competing for ^{125}I -Ang IV binding to bovine adrenal membranes (approx. 1-5nM), and ^{125}I -KYLPGPLQ (SEQ ID NO:3) displayed identical binding patterns to ^{125}I -Ang IV, whereas ^{125}I -LVVYPWTQRF (SEQ ID NO:2) bound to a subset of ^{125}I -Ang IV binding sites.

LVVYPWTQRF (SEQ ID NO:2) is released from β -globin into the circulation in uremic patients undergoing haemofiltration, and in patients undergoing open-heart surgery or cardiopulmonary bypass. Significant levels of the decapeptide are also found in ventricular cerebrospinal fluid of patients with intracerebral and subarachnoid bleeding. The half-life of the decapeptide is between 20-30min. This decapeptide may exert a modulatory effect on the activity of AT_4 receptor/IRAPs in the circulatory and central nervous systems.

30 EXAMPLE 8 IDENTIFICATION AND DEVELOPMENT OF OLIGONUCLEOTIDE MODULATORS OF IRAP/ AT_4 RECEPTOR ACTIVITY

We have shown that the levels of the AT_4 receptor are down-regulated *in vivo* by the use of anti-sense technology. Anti-sense oligonucleotides to the human sequence of AT_4 receptor are designed to bind to the specific mRNA and target the mRNA for degradation or the

prevention of translation. The invention also includes the ability to specifically regulate the different amino - terminus variants of AT₄ receptor which are encoded by splice variants of the gene by targeting the start site of translation for the differentially-spliced mRNAs. We have used an RNA secondary structure prediction algorithm, mfold, (Zucker, 1989) to identify good binding sites for the oligonucleotides. We also carried out RNA walk of the IRAP mRNA to identify regions of the RNA that contained assymmetrical bulges, short stems and included loop structures based on the predicted secondary structure of the RNA. Examples of some of the oligonucleotides identified using these methods are listed below. SEQ ID NOs:8 to 10 are oligonucleotides targeted to the mRNAs encoding both forms of the protein. SEQ ID NOs:12 and 13 are specific for the mRNAs encoding the different forms of the protein.

	5'CCAAGTAAGTGCTCATCTTCACACTCTC 3'	*	SEQ ID NO.8
20	5'GTGTTACTGTCATACAGAAGTGTCTCCTCTC 3'	*	SEQ ID NO.9
	5'CAGTTTCATGGCAGTAGTAGAGCAGTTC 3'	*	SEQ ID NO.10
	5'CATTGGTGAAGGGCTCCATTTCTTACAGCCGG 3'	*	SEQ ID NO.11
	5'CTTCCCTCAAACATGCTGTTTTCAATCATATTCCTGG 3'	*	SEQ ID NO.12
	5'TGGAGGAGGATGAAGAGGATTATG 3'	*	SEQ ID NO.13
25	5'CTACGCTATGAACTCAGCCTACACCC 3'	*	SEQ ID NO.14
	5'GCCTACACCCGAACCTAACCTCGATGA 3'	*	SEQ ID NO.15
	5'CACAGGTCATAATATTTCAAGAGT 3'	*	SEQ ID NO.16
	5'AAGCCAAGAAAAACAAGCTGAGATCC 3'	*	SEQ ID NO.17
	5'ATAAGGGATGAGCAATAC 3'	*	SEQ ID NO.18
30	5'CACCGCTTTATCAAATATGCCTAAG 3'	*	SEQ ID NO.19
	5'ATATGCCTAAGAAGTCATCAGTCGT 3'	*	SEQ ID NO.20
	5'CTACTTTGAAATTCAGTACCCACTT 3'	*	SEQ ID NO.21
	5'TTCAGTACCCACTTAAGAAATTGGAT 3'	*	SEQ ID NO.22
	5'TCACCTTCCGAGAGGAGACACTTCT 3'	*	SEQ ID NO.23

EXAMPLE 9 DEVELOPMENT OF An ASSAY FOR MONITORING THE
ACTIVITY OF AT₄ RECEPTOR/IRAP

An assay may be used for the discovery of
5 compounds which modulate the AT₄ receptor/IRAP activity.
This assay may also be used for the discovery of compounds
for the clinical applications listed herein. The assay
uses an aminopeptidase substrate, a source of AT₄
receptor/IRAP - containing material, and a candidate
10 modulator.

1. *Providing AT₄ receptor/IRAP or AT₄ receptor/IRAP*
containing material

Potential sources of AT₄ receptor/IRAP-containing
15 material include cell lines or biological tissues
expressing appreciable amounts of AT₄ receptor/IRAP.
Prokaryotic or eukaryotic cells expressing a transfected
gene encoding AT₄ receptor/IRAP represent a recombinant
source. In one embodiment of the invention, it is
20 preferable to utilize material containing AT₄ receptor/IRAP
which is identical to the form of AT₄ receptor/IRAP
expressed in the brain. Our results indicate that the
brain AT₄ receptor/IRAP is approximately 10kDa smaller than
the one expressed in the periphery. The difference in
25 size is thought to be due to differential glycosylation.
Using the brain-derived AT₄ receptor/IRAP as a source for
screening may enable the design of modulators that are
specific to the brain form of AT₄ receptor/IRAP and
therefore do not interact with the peripheral form. For
30 example, this would be important for the design of drugs
for the enhancement of memory and learning and for the
treatment of dementia.

2. *Monitoring the enzymatic activity of IRAP in the*
35 *presence of a test substance*

AT₄ receptor/IRAP-containing material is
incubated with a synthetic aminopeptidase substrate, such

as L-leucyl β -naphthylamide, and a test substance. Inhibition or enhancement of the rate of degradation of the synthetic substrate is a measure of the modulatory effect of the test substance.

5 For example, Hek293 cells stably transfected with a eukaryotic expression vector expressing the AT₄ receptor gene may be utilized. An example of this is the vector pcDNA-3 containing the cDNA encoding the full length AT₄ receptor. Two assay systems are used, one for each
10 spliced variant of the human AT₄ receptor gene. The assay system involves the measurement of the rate of hydrolysis of L-leucyl β -naphthylamide by the AT₄ receptor. The rate of hydrolysis by the transfected cells is followed by recording the increase in fluorescence (excitation at
15 340nm, emission at 410nm) using a purpose-designed, fluorimeter. Test compounds are added to the assay system, and their effects on the hydrolysis of L-leucyl β -naphthylamide are a direct indicator of their modulation of AT₄ receptor activity.

20

EXAMPLE 10 PEPTIDE INHIBITORS OF IRAP/AT₄ RECEPTOR
ACTIVITY

We believe that small peptides which bind specifically to the AT₄ receptor to mediate effects on
25 learning and memory act by inhibiting the enzymatic activity of IRAP.

Crude membranes were prepared from HEK 293 cells stably transfected with pcDNA-3 containing the cDNA encoding the full length AT₄ receptor/IRAP, using the same
30 method as that described for bovine adrenal membranes. The membranes were solubilised in 50 mM Tris-HCl containing 1% Triton-X100 at 4 °C for 16h. The IRAP enzymatic activity was determined by the hydrolysis of a synthetic substrate, L-leucine- β -naphthylamide, and
35 monitored by the release of a fluorogenic product on a spectrophotometer set at excitation 340 nm and emission 410 nm. One hundred μ M of the substrate was added to 10 μ g

of solubilised membranes, and the increase in fluorescence monitored for 10 min. The peptides Ang IV or LVVYPWTQRF (SEQ ID NO:2) (10 μ M) were added, and the extent of inhibition of enzymatic activity determined.

5 In this assay, 10 mM of Ang IV or LVVYPWTQRF (SEQ ID NO:2) totally abolished the hydrolysis of L-leucine- β -naphthylamide by membranes containing high concentrations of IRAP, as shown in Figure 12a. This indicates that in addition to binding to IRAP, these peptides inhibit the
10 catalytic activity of the enzyme.

All the AT₄ ligands tested inhibited the cleavage of the synthetic substrate, L-leucine- β -naphthylamide, by IRAP, with IC₅₀ values between 200 nM and 4 μ M, as summarised in Table 1. The rank order of potency was Ang
15 IV > Nle¹-Ang IV > LVV-hemorphin-7 > divalinal-Ang IV. In contrast, AVP, which was readily cleaved by IRAP, displayed an IC₅₀ > 40 μ M. IRAP activity could also be inhibited by the aminopeptidase inhibitors, amastatin and bestatin, but only at concentrations much higher than
20 observed for other aminopeptidases (25% inhibition of IRAP at 100 μ M amastatin, 60% at 10 μ M bestatin).

Table 1 - Comparison of the apparent affinities of AT₄ ligands, as assessed by radioligand displacement and
25 fluorescent substrate cleavage inhibition assays.

	IC ₅₀ binding ¹²⁵ I-Nle-Ang IV	IC ₅₀ inhibition leucine β - naphthylamide
Nle ¹ -Ang IV	3.32 nM	600 nM
Ang IV	32.1 nM	200 nM
LVV-H7	140 nM	1.5 μ M
Divalinal -Ang IV	845 nM	4 μ M

Kinetic analysis was performed over a range of substrate concentrations (15.6 μ M - 1 mM), in the absence

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and presence of Ang IV (0.25, 0.5, or 1 μ M) or divalinal-Ang IV (2.5, 5, or 10 μ M). Samples were assayed 2-3 times in triplicate, except controls, which were performed 5 times in triplicate. At the highest concentration of each inhibitor, cleavage of 15.6 μ M L-leucine- β -naphthylamide was essentially undetectable; therefore these samples were omitted from analysis. Linear regression analysis of kinetic data expressed in double-reciprocal (Lineweaver-Burk) form resulted in R values greater than 0.99 for each curve. Inhibitor constants (K_i) for competitive inhibitors were calculated from the relationship $IC_{50} = K_i(1 + [S]/K_m)$, where K_m for L-leucine- β -naphthylamide was determined from the kinetic experiments to be 32.3 μ M.

The results of kinetic analysis of the inhibition of IRAP by Ang IV and divalinal-Ang IV are shown in Figure 12b. Both these peptides displayed competitive inhibition of IRAP, in that the apparent K_m for L-leucine- β -naphthylamide increased up to 14-fold with increasing inhibitor concentrations, with only minor changes in V_{max} (< 30% decrease from control value of 450 pmol/ μ g protein/hr). K_i values for Ang IV and divalinal Ang IV were calculated to be 113 nM and 2.25 μ M, respectively. Nle¹-Ang IV and LVV-H7 were also competitive inhibitors; their respective K_i values were 340 nM and 845 nM.

EXAMPLE 11 DEVELOPMENT OF PEPTIDOMIMETIC AGENTS TO
MODULATE THE ACTIVITY OF AT₄ RECEPTOR/IRAP

Therapeutic agents which modulate the enzyme are also developed on the basis of structure-function studies on peptides known to modulate AT₄ receptor/IRAP. These peptides include Ang IV, Nle¹-Ang IV, LVVYPWTQRF (SEQ ID NO:2) and the complexin fragment (KYLPGPLQ (SEQ ID NO:3)), known ligands of AT₄ receptor/IRAP such as oxytocin and vasopressin, and peptide fragments thereof, and known inhibitors of aminopeptidases, enabling the development of peptidomimetic and other compounds that modulate AT₄ receptor/IRAP activity. Structure-activity studies carried

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out on amino- and carboxy-terminal extensions and deletions of the peptides Ang IV and LVVYPWTQRF (SEQ ID NO:2) using the AT₄ radioreceptor assay and 125I-Ang IV revealed that the VYXX motif is important for binding to AT₄ receptor/IRAP, as shown in Figure 13. Lead peptidomimetic compounds based on this structural backbone are being developed.

EXAMPLE 12 DEVELOPMENT OF A DIAGNOSTIC MARKER

During pregnancy AT₄ receptor/IRAP is highly expressed in the placenta. The protein is cleaved from the cell surface and circulates in the maternal serum. The serum levels of the AT₄ receptor/IRAP can be determined using a radioimmunoassay. Serum and/or CSF levels of AT₄/IRAP have the potential to be a diagnostic or prognostic marker for disorders related to the actions of AT₄ receptor/IRAP. Examples of these include central nervous system disorders, including cognitive, motor or sensory disorders such as Alzheimer's disease; motor neuron disease; cardiovascular disorders, including hypertension, congestive heart failure, cardiac hypertrophy, vasospastic disorders, and atherosclerosis; and premature or delayed labour.

EXAMPLE 13 IDENTIFICATION OF THE ENDOGENOUS SUBSTRATE/S OF AT₄ RECEPTOR/IRAP

This example relates to a method of identifying and characterising peptides that are altered by the AT₄ receptor/IRAP system including the endogenous substrate of the enzyme, particularly in the brain.

We propose that one mode of action of modulators of AT₄ receptor/IRAP is via extending the half-life of peptide substrates, by inhibiting their degradation by AT₄ receptor/IRAP. The endogenous substrates for AT₄ receptor/IRAP are identified, and their relationship to the clinical disorders and biological effects of modulators of the AT₄ receptor/IRAP system is determined.

Therefore another approach for the treatment of these disorders is to identify the targets of the AT₄ receptor/IRAP system, and to test these compounds directly, using bioassays such as memory, learning, wound
5 healing, neuronal regeneration etc.

Based on the structure of known peptide modulators for AT₄ receptor/IRAP a panel of peptides is designed, synthesise and tested as potential peptide substrates for AT₄ receptor/IRAP. Based on the ability of
10 the peptides to be altered by the AT₄ receptor/IRAP system, selective intervention such as specific blockade at the receptor level, is tested in biological systems where Ang IV and other modulators of AT₄ receptor/IRAP activity have a known effect. Therefore we can identify the peptide
15 substrates which are directly involved in the biological systems affected by the AT₄ receptor/IRAP system.

For example, a peptide is incubated with AT₄ receptor/IRAP containing material and its rate of degradation measured by HPLC. If the peptide proved to be
20 a substrate in the AT₄ receptor/IRAP system then potentially effects of modulators of AT₄ receptor/IRAP observed *in vivo* and *in vitro* may be mediated by the thus-identified substrate peptide. To confirm this hypothesis, the substrate peptide is tested in biological systems
25 where effects of Ang IV and its analogues have been observed. Alternatively, if the peptide is known to be part of an already characterised system, antagonists to the corresponding receptor are utilized to define the observed effects with Ang IV and other modulators of AT₄
30 receptor/IRAP activity.

Two candidate substrates for AT₄ receptor/IRAP in the brain are arginine-vasopressin (Herbst et al, 1997) and oxytocin (Rogi et al, 1996), two structurally related peptides which occur in relatively high abundance in the
35 brain and have been shown to be cleaved by IRAP *in vitro*. These peptides have been shown to have effects on memory in certain behavioural paradigms, and thus present two

potential substrates for IRAP in neurones. Other candidate substrates of IRAP include somatostatin, angiotensin III, Lys-bradykinin, neurotensin, met-enkephalin, dynorphin A, neurokinin A, neuromedin B, and cholecystokinin 8 (Matsumoto et al, 2001), all of which are neuropeptides which play important roles in the central nervous system.

Each potential peptide substrate (30 µg) was incubated at 37°C in TBS with 10 µg solubilized membrane protein from hIRAP transfected-HEK 293T cells. At each time point (0, 0.5, 1, 2, 4 & 6 hr), an aliquot containing 5 µg peptide was removed, and the reaction stopped by addition of 4 volumes methanol/1% trifluoroacetic acid (TFA). Samples were dried on a centrifugal vacuum evaporator prior to HPLC analysis with on-line mass spectrometric detector. Samples were loaded on to a Zorbax Eclipse C18 column (maintained at 50°C) in 1.8% acetonitrile/0.1% TFA/0.02% acetic acid at 0.15 ml/min, and eluted with a 30 minute linear gradient to 60% acetonitrile/0.1% TFA. Fragments were identified following mass spectral analysis, using Agilent ChemStation deconvolution software. The results are summarised in Table 2.

Among the range of potential substrates tested, arginine-vasopressin (AVP), oxytocin (OXY) and met-enkephalin were the most rapidly cleaved by IRAP. Both Cyt⁶-AVP(2-9) and Cyt⁶-AVP(3-9) were generated by IRAP, but only the first peptide bond of OXY was cleaved. Similarly, although the first residue (Tyr¹) of met-enkephalin was quickly removed, only a small amount of desTyr¹Gly²-met-enkephalin was detected after 6 hours. This contrasts with the longer opioid peptide α-neo-endorphin (10 residues), in which at least three N-

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terminal residues were cleaved, although less rapidly than met-enkephalin. Furthermore, α -endorphin, which is 16 residues long, with the first five residues identical to met-enkephalin, was not cleaved at all, suggesting that peptide chain length is an important determinant of substrate specificity and efficiency. The only other peptide to be cleaved by IRAP was neurotensin, despite the presence of a pyroglutamyl residue at the N-terminus in this peptide.

10 Among the angiotensin peptides, no cleavage was observed for Ang I, Ang II, or divalinal-Ang IV. No cleavage of the other peptides tested (GnRH, acetylated AVP, and [pGlu⁴Cyt⁶]AVP(4-9)) was observed.

Table 2 - Degradation of Ang IV-related and other peptides by IRAP expressed in HEK 293T cells.

<u>Peptide</u>	Products Identified	Substrate disappearance over 4 hr
AVP	YFQNCytPRG-NH ₂ FQNCytPRG-NH ₂	58%
OXY	YIQNCytPLG-NH ₂	37%
Met-enkephalin	GGFM	65%
Neurotensin	LYENKPRRPYIL	22%
Ang IV	YIHPPF	15% (= UT)
Nle ¹ -Ang IV	YIHPPF	16% (= UT)
LVVYPWTQRF	VVYPWTQRF	14% (= UT)
Ang III	RVYIHP	7% (= UT)
α -neo-Endorphin	GGFLRKYPK, GFLRKYPK, FLRKYPK	9%
Divalinal Ang IV	None	None
Ang I	None	None
Ang II	None	None
Sar ¹ Ile ⁸ -Ang II	None	None
GnRH	None	None
AcAVP	None	None
pGlu ⁴ Cyt ⁶ AVP	None	None
α -Endorphin	None	None

5 UT : untransfected cells used as the control

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In adipocytes and skeletal muscles, IRAP is found in the same vesicles as GLUT4, the insulin-dependent glucose transporter. As in the case of GLUT4, IRAP is redistributed to the cell surface in response to insulin or insulin-like growth factors. We postulate that IRAP may serve as a negative feedback control for GLUT4 translocation to the cell surface and hence glucose transport into the cell by regulating the levels the insulin and insulin-like growth factors on the cell surface and also in the GLUT4 vesicles. We are investigating the hydrolysis of insulin IGF-I and IGF-II by IRAP using HPLC with C18 reverse phase columns.

15 EXAMPLE 15 EFFECTS OF INHIBITORS/SUBSTRATES OF AT₄
RECEPTOR/IRAP ON ANIMAL MODELS OF MEMORY AND
LEARNING

Previous studies have shown that binding of peptide agonists to the AT₄ receptor in normal animals led to improved performance of memory tasks in both passive avoidance and spatial learning paradigms, and reverses memory deficits induced chemically by muscarinic antagonist or mechanically by knife-cut lesions. The initial effects were observed in the passive avoidance paradigm in rats where an intracerebroventricular dose (1 nmol) of Ang IV increased the latency in re-entering the dark chamber after an aversive stimulus (Braszko et al, 1988, Wright et al, 1993, Wright et al, 1996). More recent studies revealed that a more stable analogue of angiotensin IV, Nle-angiotensin IV, given acutely into the lateral ventricles, reversed the memory deficits induced by the muscarinic receptor antagonist, scopolamine in a spatial learning paradigm (Pederson et al, 1998). Chronic infusion (6 days) of this analogue into the lateral ventricle of rats at a dose of between 0.1 and 0.5 nmol/h enhanced performance in the swim maze task. This effect was blocked by divalinal angiotensin IV, suggesting that

this was an angiotensin AT₄ receptor mediated effect (Wright et al, 1999). In this swim maze paradigm, memory deficits induced by bilateral perforant pathway lesion can be reversed by an acute dose (1 nmol) of another AT₄ receptor agonist, Norleucinal angiotensin IV (Wright et al, 1999).

In the context of this invention, modulation of the activity of the aminopeptidase AT₄ receptor/IRAP by a number of peptides known to be AT₄ receptor agonists results in enhancement of learning and memory in normal and amnesic animals.

EXAMPLE 16 EFFECTS OF MODULATION OF AT₄ RECEPTOR/IRAP ON SPATIAL AND LEARNING MEMORY IN NORMAL RATS

Normal male Sprague-Dawley rats were implanted with cannulas in the lateral ventricles and allowed to recover for at least three days. The animals were subjected to a modification of the Barnes Circular Maze test (Barnes, 1979). The maze comprised a raised rotatable white circular platform of diameter 1.2m, with 18 evenly spaced holes in the periphery (diameter 0.09m). An escape tunnel comprising a black box of internal diameter of 0.16m width, 0.29m length and 0.09m depth was positioned immediately beneath a peripheral hole. The maze was positioned in a large room, 6 × 4.5m, both naturally and fluorescently lit with visual cues (e.g. A4 sized posters of abstract patterns) placed on stands at various positions around the maze at a distance of 0.5m from the platform.

For each trial, the animal was placed on the maze platform under the starting chamber, which is a cylindrical chamber located in the centre of the platform and can lowered or raised, and left for 20s. While the animal was under the chamber, the floodlights were switched on. Following the twenty seconds disorientation period the chamber was raised, and the trial was begun. The animals were then allowed 240s in which to find and

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utilize the escape tunnel. Following 20s of light alone, the auditory buzzer was switched on, and these aversive stimuli were continued throughout the remaining time period (220s) or until the escape tunnel was found and
5 utilized. Each rat received three consecutive trials per day over ten days. On the first day of the testing period, each rat was individually taken into the testing room in its cage, and placed directly into the escape tunnel for a 2min familiarization period. The rat was then
10 replaced into its cage for approximately one minute, after which the animal was injected with peptide or artificial cerebrospinal fluid and then replaced into its cage for a further 5min. Three consecutive trials were then carried out, with a recovery period of 2min between each trial in
15 its home cage.

The escape tunnel and platform were cleaned to eliminate the possible use of olfactory cues in the next trial. The platform was then rotated so that a different peripheral hole was placed above the escape tunnel
20 although the spatial location of the escape tunnel is kept constant for each animal. On subsequent days (days 2 to 10), the familiarization period prior to the three trials was eliminated from the protocol.

In the Barnes maze, treatment with 100pM or 1nM
25 of Nle¹-Ang IV or LVVYPWTQRF (SEQ ID NO:2), both competitive inhibitors of IRAP, decreased the time taken to achieve learner criteria in this paradigm. The results are illustrated in Figures 14a and 14b. The learner criterion is dependent on the number of errors made per
30 trial and the time taken to reach the escape tunnel for each trial. Control animals treated with artificial cerebrospinal fluid took 7 days to achieve learner criteria, whereas animals treated with Nle¹-Ang IV or LVVYPWTQRF (SEQ ID NO:2) at a concentration of either
35 100pM or 1nM LVVYPWTQRF (SEQ ID NO:2) managed this in 3-4 days (Figure 14a). Moreover, during each trial, even from day 1 of testing, the peptide-treated animals consistently

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made fewer errors per trial, and travelled shorter distances to find the escape tunnel than the artificial cerebrospinal fluid control. This observation strongly indicates that the two peptides tested not only improved
5 memory, but also enhanced the ability of the animals to learn.

EXAMPLE 17 EFFECT OF MODULATION OF AT₄ RECEPTOR/IRAP
ACTIVITY ON SPATIAL MEMORY IN NORMAL MICE

10 The effect of modulation of AT₄ receptor/IRAP activity on spatial memory in normal mice was then tested using the Y-maze test. This test is another behavioural paradigm which measures spatial memory performance, and exploits the natural instinct of rodents to explore novel
15 environments. This test is less stressful than other behavioural paradigms, including Morris water maze and passive avoidance tasks, which can modify the motivational and emotional status of the animals.

 The Y maze consists of three identical alleys
20 (30cm in length, 10cm in width and 17cm in height) with the three arms separated by 120° angles. To minimize stress, the maze is located in a sound-attenuated room under dim illumination, with the floor of the maze covered with sawdust. After each trial, the sawdust is mixed to
25 eliminate olfactory cues. For spatial orientation, visual cues are placed on the walls of the testing room.

 The test consisted of two trials, separated by a time interval known as the intertrial interval. During the acquisition trial, the animals were placed at the distal
30 end of one arm, their heads pointing away from the centre of the maze. The mice were allowed to visit only two accessible arms of the maze for 3 minutes. At the end of the acquisition trial, the mice were replaced in their cages for the intertrial interval. During the retention
35 trial, the animals had access to all three arms for 5 minutes. The number and duration of time spent in the novel arm were documented.

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A shorter intertrial interval (2 hours) separating the acquisition phase from the retention phase enables amnesiant effects to be detected. Control mice still remember the location of the novel arm and will
5 preferentially spend more time (45-50% of time) in that arm. A longer intertrial interval (6 hours) results in the animals not remembering the location of the novel arm and hence spending equal amount of time in all three arms.

Mice were surgically implanted with an infusion
10 cannula in the dorsal third ventricle. The cannula was connected to Alzet osmotic minipumps by tygon tubing (5-6cm in length and 0.3mm in diameter). The minipumps were placed in the dorsal subcutaneous space in the neck. The minipumps were filled with artificial cerebrospinal fluid
15 for 1 week, and delivered the fluid at a constant flow rate of 0.5µl/h. At the end of the first week, the minipumps were replaced under light anaesthesia with another minipump containing either artificial cerebrospinal fluid or LVVYPWTQRF (SEQ ID NO:2). The
20 peptide was delivered at a rate of 0.3pmol/h.

Each mouse was tested three times, and each test was separated by at least three days. The longer intertrial interval regime was adopted to test for memory-enhancement potential of the peptide. The first test was
25 performed prior to implantation of the minipumps with treatment, and hence constituted measurement of the basal memory potential of the animals. The second and third tests were carried out on days 3 and 6 of treatment delivery. The results are summarised in Figure 15.

30 After three days of treatment, mice which received LVVYPWTQRF (SEQ ID NO:2) spent more time in the novel arm (between 50-60% of time) compared to control mice (approximately 33% of time), indicating that during that time period, LVVYPWTQRF (SEQ ID NO:2) enhanced the
35 spatial memory in this paradigm. However, after 6 days mice from both treatment groups were indistinguishable from each other. These results indicate that prolonged

treatment with LVVYPWTQRF (SEQ ID NO:2) is detrimental to spatial memory, and may be the result of excessive accumulation of the peptide in the hippocampus.

5 EXAMPLE 18 EFFECT OF MODULATION OF AT₄ RECEPTOR/IRAP
 ACTIVITY ON SPATIAL LEARNING AND MEMORY IN
 AMNESIC ANIMALS

 The effect of modulation of AT₄ receptor/IRAP
 activity on spatial learning and memory in amnesic animals
10 was also assessed using the Morris water maze test. The
 Morris test has been used extensively to test for spatial
 memory. The hippocampus, which contains high levels of AT₄
 receptors/IRAP, is thought to play an important role in
 processing this form of memory.

15 Scopolamine, a muscarinic receptor antagonist,
 has been used to induce amnesia in a number of behavioural
 paradigms of memory and learning. This compound has been
 shown to block the trial-to-trial decrease in latency of
 this task, and this effect appeared to be due to
20 impairment of short-term memory. The effect of AT₄
 receptor/IRAP ligands on scopolamine-induced amnesia in a
 spatial memory task was tested in the Morris water maze.

 The maze consisted of a circular tank, 1.6m in
 diameter by 0.6m in height, containing water to a depth of
25 30cm. The room contained visual cues placed 40 cm from
 the edge of the tank. The rats were subjected to two days
 of familiarisation to the task, during which the escape
 pedestal is visible to the animals. During the
 acquisition phase, for the following 5 days, the water in
30 the tank was rendered opaque, and the escape platform was
 submerged under the surface of the water. The animals had
 to rely on the visual cues around the room to find the
 submerged escape pedestal. The latency of the animal in
 finding the pedestal during the acquisition phase was
35 taken as a measure of the spatial memory performance of
 the animal.

 Rats were surgically implanted with cannulae in

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their lateral ventricles and allowed two to three days of recovery. During the familiarisation trials on days 1 and 2, the animals were placed in the water facing the wall of the tank from different starting positions equidistant from the escape pedestal. When the animals located and mounted the pedestal, they were allowed 30 seconds on the pedestal before the commencement of the next trial. Four consecutive trials were carried out on each animal on each day, and the mean latency period before the animal reached the pedestal was plotted.

For the acquisition trials, from days 3-7, the animals were randomly divided into three groups:

- a) saline control,
- b) scopolamine followed by saline treatment, and
- 15 c) scopolamine followed by LVVYPWTQRF (SEQ ID NO:2) treatment.

The scopolamine-treated animals were given 70nmole of the compound into the lateral ventricles 30min before the commencement of the trials for each day. The peptide (1nmole) or saline was given 5min before the trial began. The pedestal, which remained in a constant position for each animal, was submerged for this phase, and the mean latency for each animal in locating the pedestal was documented for each day. The drug treatments were withdrawn on the eighth day, the pedestal once again made visible, and latency documented. The results are shown in Figure 16.

Intracerebroventricular infusion of 70nmole of scopolamine significantly increased the latency of the animals in locating the submerged pedestal, indicating a deficit in short-term memory. Rats treated with LVVYPWTQRF (SEQ ID NO:2) reversed the scopolamine-induced latency, and this group of rats was indistinguishable from saline-treated controls. Withdrawal of treatment on day 8 brought the latency of the scopolamine-treated group back to control levels, indicating that the effect of scopolamine was reversible, and that the latency was

primarily due to the muscarinic receptor antagonist.

EXAMPLE 19 EFFECT OF MODULATION OF AT₄ RECEPTOR/IRAP
ACTIVITY ON AVERSIVE CONDITIONING BEHAVIOUR
IN AMNESIC ANIMALS

5 Passive avoidance trials were then used to test
the effect of modulation of AT₄ receptor/IRAP activity on
aversive conditioning behaviour in amnesic animals. The
passive avoidance task involves aversive conditioning
10 behaviour to measure facilitation of memory retention and
retrieval. The testing was carried out in an apparatus
that consisted of a light and a dark compartment separated
by a guillotine door. The floor of the dark compartment
contained an electrified grid.

15 Rats were surgically implanted with chronic
cannulae in the lateral ventricle. On conditioning day,
the animals were habituated in the dark compartment for
5min with the guillotine door closed. The animals were
then placed in their home cages for 5min before being
20 placed in the light compartment with the guillotine door
opened. The latency to enter the dark compartment with
all four feet was documented. When the rats entered the
dark compartment within 20s of being placed in the light
compartment, they were randomly divided into four
25 treatment groups. The treatment groups were:
(1) saline followed by saline;
(2) saline followed by 1.0nmole LVVYPWTQRF (SEQ ID
NO:2);
(3) 70nmole scopolamine followed by saline; and
30 (4) 70nmole scopolamine followed by 1.0nmole
LVVYPWTQRF (SEQ ID NO:2).

 All of these were administered into the cerebral
ventricles 30min and 5min before the acquisition trial.
During this trial, the guillotine door was closed once the
35 animal entered the dark compartment, and the rat then
received a low-level electric shock (0.2mA) via the grid
floor. The animal was then returned to its home cage for

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24h before being tested once daily for the next four days and the latency periods to re-enter the dark compartment were taken as a measure of the ability of the animals to remember the aversive stimuli. The results are shown in Figure 17.

Control rats displayed high latency periods in entering the dark compartment, due to the successful conditioning, whereas rats treated with scopolamine displayed memory deficits, as indicated by the much lower latency periods. The performance of rats which received LVVYPWTQRF (SEQ ID NO:2) after scopolamine was not significantly different from controls indicating that the decapeptide completely reversed the amnesia induced by scopolamine. Rats which received LVVYPWTQRF (SEQ ID NO:2) alone displayed latency periods that were comparable to the scopolamine-treated group. This suggests that at high doses, LVVYPWTQRF (SEQ ID NO:2) is detrimental to learning, as was observed in the Y maze paradigm.

20 EXAMPLE 20 MECHANISM OF MEMORY/LEARNING ENHANCEMENT OF
THE IRAP/AT₄ RECEPTOR SYSTEM

Acetylcholine is a major neurotransmitter involved in the processing of cognitive function, since anti-cholinergic drugs induce memory deficit and confusion. In Alzheimer's disease, neuronal loss has been reported in cholinergic-rich areas, particularly in the septohippocampal pathway. Angiotensin AT₄ receptors have been found in high abundance in the basal nucleus of Meynert, in the CA2 and dentate gyrus of the hippocampus, and in somatic and autonomic preganglionic motoneurons of the monkey brain. This pattern of receptor distribution closely resembles that of cholinergic neurones and their projections, and suggests that the AT₄ receptors may be associated with cholinergic pathways centrally. Moreover, as shown in Example 19, LVV-haemorphin-7 can reverse the learning deficit induced by scopolamine (a muscarinic receptor antagonist). We therefore postulated that

LVVYPWTQRF (SEQ ID NO:2) can modulate acetylcholine release from the septohippocampal neurones via the AT₄ receptors. In the light of:

- (a) our discovery that IRAP is the AT₄ receptor;
 - 5 (b) the possibility that IGF or insulin is a substrate for IRAP; and
 - (c) the fact that glucose has been shown to facilitate acetylcholine synthesis and release
- we believe that the AT₄ receptor/IRAP may be regulating
- 10 glucose uptake into the hippocampus resulting in an increase in acetylcholine release.

To determine the effect of IRAP inhibitors on acetylcholine release for rat hippocampal slices *in vitro*, rats were anaesthetized with sodium pentobarbitone,

15 decapitated and the brain rapidly extracted and put on ice. The two sides of the hippocampus were then dissected, and stored in Krebs buffer on ice. The tissues were sectioned into 400µm slices using a McIlwain slicer, and incubated with 10µM of ³H-choline in Krebs buffer for

20 20min at 37°C. Following the incubation, the sections were transferred to Brandel superfusion chambers filled with carbogenated Krebs buffer, pH7.0 containing 50mM hemicholinium HC-3 to prevent choline reuptake. The

25 slices were washed for 60min at 37°C with Krebs buffer at a flow rate of 500µl/min. Superfusate samples were collected at 5min intervals for the duration of the experiment. After a stable baseline was established, the hippocampal slices were depolarised by the addition of 30mM KCl to the Krebs buffer and infused for 4min (stimulation period S1).

30 The tissue was then allowed to recover for 25min before the next stimulation period (S2). To test the effect of LVVYPWTQRF (SEQ ID NO:2) or Ang IV on the evoked release of ³H-choline, the peptides were added to the hippocampal slices 15min prior to and 5min during the second period of

35 stimulation. At the end of the experiment, tissues were collected and solubilized with 2ml of solubene to determine the counts remaining in the hippocampal slices.

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Fractional ^3H -choline release, expressed as a ratio of S2/S1, was then calculated for each of the different peptides at the different doses.

In rat hippocampal slices, LVVYPWTQRF (SEQ ID NO:2) significantly and dose-dependently increased potassium-evoked ^3H -choline release, with a maximum effect of 180% of control observed at 10^{-7}M , as shown in Figure 18. Similarly, Ang IV, also significantly and dose-dependently increased potassium-evoked ^3H -choline release with a smaller maximum effect of 150% of control observed at 10^{-7}M , as shown in Figure 18.

EXAMPLE 21 EFFECT OF AT_4 RECEPTOR/IRAP LIGANDS ON AND
MAP KINASE, CREB AND ELK-1 PHOSPHORYLATION

Long-term neuronal plasticity and memory consolidation require *de novo* gene expression which is thought to be mediated by the activation of certain transcription factors, such as the cAMP response element binding protein (CREB) and Elk-1 (a transcriptional regulator of serum response element-driven genes), via the ERK/mitogen activated protein kinase (MAPK) signalling cascade. The ERK/MAPK cascade involves three tiers of kinases which are sequentially activated by phosphorylation. The MAP kinase kinase kinases, Raf-1 and B-Raf, activate the MAP kinase kinase, MEK, by serine/threonine phosphorylation; MEK then activates p44 MAPK and p42 MAPK, by threonine and tyrosine phosphorylation. Phosphorylation of these MAPKs is thought to bring about their translocation to the nucleus, where they alter gene expression via activation of CREB and Elk-1. MAPK is also thought to regulate the voltage-dependent activation of the potassium channel, Kv4.2, which plays a key role in regulating calcium influx. The MAPK cascade is activated by multiple signals, including activation of receptor tyrosine kinases, synaptic activity and increases in intracellular Ca^{2+} and cAMP, and is thus capable of

integrating several different signals into a graded response.

The mechanism(s) by which IRAP/AT₄ substrates enhance memory may involve modulation of the MAPK cascade. For instance, inhibition of the aminopeptidase activity of IRAP/AT₄ by ligand binding might bring about the accumulation of a neuropeptide that activates the MAPK cascade via an interaction with a cell surface receptor.

The potential of IRAP/AT₄ ligands to activate MAPK or CREB was initially investigated on rat hippocampal slices. Rats were killed by decapitation and the hippocampi removed, four hundred μ m thick slices cut and incubated in aCSF. After four hours of stabilisation, the slices were treated with 10^{-8} M Nle¹Ang IV for 30 min, or left untreated. Slices were then lysed in 200 μ l of ice-cold lysis buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1% triton X-100, 30 mM sodium pyrophosphate, 50 mM NaF, 5 μ M ZnCl₂, 0.1 mM sodium orthovanadate, 1mM DTT, 5 nM okadaic acid, 1 x Boehringer Protease inhibitor cocktail) and centrifuged at full speed. The lysates were then heated at 95°C for 10 min in 6 x SDS loading buffer and the proteins (30 μ g per lane) resolved on 7.5% SDS-poylacrylamide gels. After transfer of proteins to PVDF membranes, blots were probed overnight at 4°C with anti phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody and detected by chemiluminescence using ECL blotting detection reagents and a Fuji LAS-1000.

A single dose of 10^{-8} M Nle¹-Ang IV induced phosphorylation of ERK1/2 in rat hippocampal slices after 30 min, as shown in Figure 19a. This effect was observed throughout the length of the hippocampus.

Enhanced activation of the MAPK cascade by IRAP/AT₄ ligands may only occur during learning. Therefore, animals treated IRAP/AT₄ ligands or vehicle control are subjected to varying numbers of learning trials (as described in the previous example), before analysis of the

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levels of phospho-MAPK phospho-CREB and phospho-Elk-1 in their hippocampi as described above.

Alternatively, binding of ligands to IRAP might generate an intracellular signal which activates the MAPK cascade. The ability of AT_4 ligands to stimulate MAPK phosphorylation directly was investigated in HEK293 cells transfected with human IRAP. HEK293 cells were transfected with pCI-IRAP or with empty vector, plated on to 12-well plates at a density of 60,000 cells/well and grown in complete medium containing 0.2% fetal calf serum. The cells were then treated with 10^{-6} , 10^{-9} or 10^{-11} M Nle^1 -Ang IV for 5, 30 or 120 min. After peptide treatment, the medium was removed, the cells washed in phosphate-buffered saline and solubilised in 100 μ l of 1xSDS loading buffer. The solubilised samples were then heated at 100°C for 5 min, centrifuged and 50 μ l of the supernatant run on a 12% SDS-PAGE. The proteins were then transferred on to nitrocellulose membranes and incubated with phospho-MAPK (p44/42) antibodies and visualised with HRP-conjugated secondary antibody, using the ECL-plus western blot detection system.

Nle^1 -Ang IV at concentrations of 0.001, 1 and 10 μ M, induced the phosphorylation of ERK1/2 within 5 min of peptide treatment, and the phosphoERK persisted for 2 h, as shown in Figure 19b. The largest response was observed at 0.001 μ M of Nle^1 -Ang IV.

EXAMPLE 22 IRAP COLOCALISES WITH GLUT4 IN NEURONES -
COMPLEMENTARY ROLE IN THE REGULATION OF
GLUCOSE UPTAKE IN NEURONAL CELLS

We have also investigated the role of AT_4 receptor/IRAP in the regulation of glucose uptake into neuronal cells. GLUT4 was initially found predominantly in adipocytes and skeletal muscles, where it is highly expressed in vesicles. Activation of the insulin receptor by insulin or insulin-like growth factors in these cells results in the translocation of GLUT4 vesicles to the

plasma-membrane. This insulin-dependent glucose transporter was recently detected in the brain and in developing mouse embryos. In the brain, the distribution of GLUT4 protein and mRNA closely parallels the location of the AT₄ receptor, as demonstrated by ¹²⁵I-Ang IV or ¹²⁵I-Nle Ang IV binding. Since IRAP is the AT₄ receptor, we proposed that, as in adipocytes and skeletal muscles, GLUT4 is co-localised with IRAP in neurones, and subserves a similar role.

We investigated the cellular localisation of IRAP, using an antibody raised in rabbits against a GST fusion protein containing the cytoplasmic tail of murine IRAP. We also investigated the cellular localisation of GLUT4, using a monoclonal antibody against mouse GLUT4.

Mice (C57/Black females) were anaesthetised with an intraperitoneal injection of pentobarbitone, and perfused with phosphate-buffered saline (PBS) followed by 4% PFA, 2% glutaraldehyde in phosphate buffer (pH 7.4). The brains were taken and stored in the fixative overnight at 4°C, then snap-frozen in isopentane cooled with dry ice. Forty micron thick sections were cut on a cryostat and collected into PBS. The free-floating sections were then incubated for one hour at room temperature in PBS with normal horse serum (10%) and Triton X-100 (0.3%). The sections were transferred to a blocking solution (NEN TSA kit) for 30min before incubation with the primary antibodies. Both antibodies (rabbit anti-IRAP and mouse anti-NeuN or rabbit anti-IRAP and mouse anti-GLUT4) were diluted 1:200 in PBS with 2% normal horse serum and 0.3% Triton X-100. The sections were incubated for 48h at 4°C.

Following incubation with the primary antibodies, the sections were washed three times in PBS and incubated for an hour at room temperature in anti-rabbit and anti-mouse IgG secondary antibodies conjugated to Texas Red (anti-rabbit) and FITC (anti-mouse). These antibodies were diluted in PBS with 2% normal horse serum. The sections were washed three times in PBS, mounted onto

gelatine-coated slides and a coverslip was added with DAKO fluorescent mounting medium. The results are shown in Figure 20.

We found, using immunohistochemical staining of mouse hippocampus with an IRAP antibody, that IRAP was present in all pyramidal cells of the CA1-CA3 region and the Purkinje cells of the cerebellum. Moreover, all cells which were immunopositive for IRAP also contained GLUT4-positive immunoreactivity. However, the distribution of IRAP in the neurones appeared punctate, and occurred throughout the cell body and its processes, whereas the GLUT4 positive staining was perinuclear and therefore not in the same cellular compartment as IRAP (Figure 20). The intracellularly distributions of the two proteins did not reflect what is observed in adipocyte and skeletal muscles. We believe that in brain, IRAP may have wider roles in the regulation of local neuropeptide concentration, in contrast to IRAP in adipose tissue and skeletal muscle, where the enzyme coexists with GLUT4, and may be integrally involved in insulin-regulated glucose transport. However, it is still possible that in neurones, IRAP is involved in the regulation of the levels of insulin or insulin-like growth factors by enzymatic cleavage of these peptides, under certain physiological conditions such as during neural activation or pathophysiological states such as cerebral ischemia. Under these conditions, IRAP may play a role in the regulation of glucose uptake in neurones.

EXAMPLE 23 EFFECT OF MODULATORS OF IRAP/AT₄ RECEPTOR
ACTIVITY ON GLUCOSE UPTAKE INTO NEURONAL
CELLS

Glucose has been found to improve memory in animals and humans, and several studies have shown that the memory-enhancing effects of glucose may not be due solely to its role in providing fuel for brain cells (Wickelgren, 1998). Glucose has been shown to facilitate

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acetylcholine synthesis (Kopf et al, 2001) and release (Kopf et al, 2001). Accordingly an investigation of AT₄ receptor/IRAP activity on glucose uptake in the hippocampus is undertaken.

5 Male Sprague-Dawley rats are surgically implanted with chronic cannulae in their lateral ventricles and allowed to recover for at least three days. To evaluate the effects of Ang IV or LVVYPWTQRF (SEQ ID NO:2) on hippocampal glucose uptake, the rats are infused with
10 1nmole of either peptide over a period of 10min. Immediately after the infusion of peptide, the rats are injected with 2-deoxy-D-[1-¹⁴C]glucose into the peritoneal cavity at a dose of 1μCi/g. The rats are killed 45min later, the brains extracted and snap-frozen and sectioned
15 at 14μm thickness and exposed to Hyperfilm βmax.

Glucose utilisation is also measured on synaptosomes prepared from hippocampus. Synaptosomes are isolated from homogenised rat hippocampus using the Ficoll gradient centrifugation technique. The synaptosomes are
20 washed in glucose-free HEPES buffer, then incubated at 37°C for 5min in the same solution containing 0.5mmole glucose. The synaptosomes are loaded with oxy-D-glucose, 2-[1,2-³H(N)] at a concentration of 1μCi/ml. Uptake is assayed in the absence or presence of Ang IV or LVVYPWTQRF (SEQ ID
25 NO:2) at concentrations of 1nM to 1μM. To terminate the experiments, the samples are centrifuged at 16000g for 30s, the supernatant discarded and the pellet rinsed twice with ice-cold HEPES buffer. The radioactivity retained is measured by scintillation counter, and expressed per mg
30 protein. Hexokinase I activity, which is responsible for glucose-6 phosphorylation and essential for neuronal glucose utilisation, is also determined on the synaptosomal preparations, since a decrease in glucose utilisation is reflected by reduced hexokinase activity.
35 Hexokinase activity is assayed on 0.2mg of freshly prepared synaptosomes suspended in MOPS-Tris buffer containing 0.1% Triton X-100, 8mM MgCl₂, 0.4M NADP⁺, 5mM D-

glucose, 5 units of glucose-6-phosphate dehydrogenase and 1mM ATP in the absence or presence of Ang IV or LVVYPWTQRF (SEQ ID NO:2). The linear rate of NADP⁺ reduction is monitored on a spectrophotometer at 340nm absorbance and expressed as nM of product formed per mg protein.

We postulate that the modulation of AT₄ receptor/IRAP activity by Ang IV or LVVYPWTQRF (SEQ ID NO:2) results in an increase in glucose uptake in the hippocampus. This may be part of the mechanism via which the two peptides act to produce the effects on acetylcholine release and facilitation of memory.

The effect of IRAP inhibitors on the regulation of glucose uptake was determined in 3T3L1 cells. 3T3L1 fibroblasts were differentiated by the addition of DMEM containing 5% fetal calf serum, 4 µg/ml insulin, 0.25 mM dexamethasone and 0.5 mM 3-isobutyl-1-methyl-xanthine. After 7-14 days, more than 90% of the fibroblasts were differentiated into mature adipocytes. These cells were then placed in DMEM containing 25 mM glucose, 0.1% bovine serum albumin, washed in HEPES buffer and then treated with insulin (0.7 or 70 nM) or buffer with or without concurrent treatment with either 10⁻⁷ or 10⁻⁶ M Nle¹-Ang IV for 30 min. Glucose uptake was determined by incubation on ice for 5 min with 100 µM 2-deoxyglucose containing 1.0 µCi of 2-[³H]deoxyglucose. The reaction was stopped after 10 min by washing the cells in PBS. The cells were then solubilized in 0.5 N NaOH and counted.

Both concentrations of Nle¹-Ang IV increased basal glucose uptake two-fold : untreated (3.3 ± 0.67 pmol/min/well) compared to 10⁻⁷ M Nle¹-Ang IV (7.2 ± 1.79 pmol/min/well) and 10⁻⁶ M Nle¹-Ang IV (7.6 ± 1.21 pmol/min/well). Under insulin stimulation, glucose uptake increased to 13.5 ± 2.6 pmol/min/well for 0.7 nM insulin and 19.7 ± 1.18 pmol/min/well for 70 nM insulin. The effect of Nle¹-Ang IV on insulin-stimulated glucose uptake was not as marked. For both the concentrations of insulin, treatment with 10⁻⁷ M Nle¹-Ang IV did not

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significantly increase glucose uptake (12.2 ± 2.29 pmol/min/well for 0.7 nM insulin and 22.99 ± 2.8 pmol/min/well for 70 nM insulin) whereas treatment with 10^{-6} M Nle¹-Ang IV did (18.3 ± 2.39 pmol/min/well for 0.7 nM insulin and 23.02 ± 1.52 pmol/min/well for 70 nM insulin).

EXAMPLE 24 EFFECT OF MODULATORS OF IRAP/AT₄ RECEPTOR
ACTIVITY ON TROPHIC EFFECTS OF INSULIN OR
INSULIN-LIKE GROWTH FACTOR

10 In addition to the role of insulin and insulin-like growth factor in chaperoning glucose into neurones via translocation of the GLUT4 vesicles, these two polypeptides could have other roles in the brain which may lead to facilitation of memory. Insulin and IGF-I have
15 been shown to stimulate neuronal growth and promote arborisation of dendrites (Bondy, 1991, Wickelgren, 1998, O'Kusky et al, 2000). Some of the memory-enhancing effects of Ang IV and LVVYPWTQRF (SEQ ID NO:2), particularly with chronic infusions, may be the result of
20 prolonging the half-life of insulin or IGF in the brain, resulting in an increase in neurogenesis or synaptogenesis.

 This ability can be assessed by measuring neurogenesis in the hippocampus of rats subjected to the
25 Barnes maze paradigm in the presence and absence of Nle-Ang IV or LVVYPWTQRF (SEQ ID NO:2), using a marker of dividing cells, 5-bromo-2'-deoxyuridine (BrdU). Male Sprague-Dawley rats are implanted with chronic intracerebroventricular cannulae and allowed two to three
30 days recovery before the commencement of the experiment. The rats are divided into three groups:

- 1) Naïve animals receiving no training on the Barnes maze;
- 2) Animals trained on the Barnes maze, receiving one
35 single infusion of artificial cerebrospinal fluid 5min before commencement of the first trial on the first day; and

3) Animals trained on the Barnes maze, receiving one single dose of 100pmol Nle-Ang IV or LVVYPWTQRF (SEQ ID NO:2) 5min before the commencement of the first trial on the first day.

5 All rats are injected with bromodeoxyuracil (BrdU; 50mg/kg) twice daily for 3 days. The two groups of rats subjected to the behavioural paradigm are tested for 3 days on the Barnes maze, commencing the day of the first BrdU injection. During this time most of the rats which
10 receive the peptide treatment are expected to achieve learner criteria, whereas the rats treated with artificial cerebrospinal fluid do not. The animals are perfused intracardially with 4% paraformaldehyde in phosphate buffered saline 24h after the last BrdU injection. The
15 brains of these animals are removed and processed for BrdU immunofluorescence using a mouse anti-BrdU primary antibody and detected with an anti-mouse secondary antibody conjugated with FITC. The number of BrdU-labelled cells are determined on at least 24 sections (14µm
20 thick) of the rat hippocampus in the rostro-caudal extent.

To determine if the increase in neurogenesis induced by Ang IV or LVVYPWTQRF (SEQ ID NO:2) is due to prolonging the half-life of insulin or insulin-like growth factor, this experiment is repeated in the presence of a
25 specific antibodies raised against insulin or IGF.

EXAMPLE 26 ROLE OF AT₄ RECEPTOR/IRAP IN TISSUE
DEVELOPMENT AND REGENERATION

AT₄ receptor ligands have been shown to induce
30 cellular proliferation in cardiac fibroblasts and neuronally-derived immortalised cell lines. This suggests that AT₄ receptor/IRAP may be abundantly expressed during development, and may have an important role in embryonic development. We investigated the expression of AT₄
35 receptors/IRAP in embryonic and neonatal mice, and compared the levels of expression with those of adult

mice. Quantitative *in vitro* receptor autoradiography and *in situ* hybridisation histochemistry was used to determine the levels of AT₄ receptor/IRAP protein and mRNA.

Embryonic day 13,15,17, neonatal day 1,7,21 and
5 adult mice were killed by isofluorane inhalation. The embryos were embedded in mounting medium and snap-frozen whole, whereas in the neonatal and adult mice, tissues were dissected out and frozen in isopentane chilled to -40°C on dry ice. For the determination of AT₄
10 receptor/IRAP protein, 10µ frozen sections were cut, thaw-mounted on to gelatine-coated slides, and dried for 2h under reduced pressure. The sections were then rinsed in buffer (pH 7.4) containing 50mM Tris, 5mM EDTA, 150mM NaCl for 30min at room temperature. This was followed by a 2h
15 incubation in the buffer described above to which had been added 100µM PMSF, 20µM bestatin, 100µM phenanthroline, 0.1% BSA and 130pM ¹²⁵I-Nle¹ Ang IV. The sections were then washed four times in Tris-buffered saline at 0°C for 2min each, dried rapidly and exposed to X-ray film.

20 For the determination of AT₄ receptor/IRAP mRNA, 10µ frozen sections were cut, thaw-mounted onto silane-coated slides and stored at -20°C. Eight oligonucleotides, four in the anti-sense orientation and four in the sense orientation, were prepared from different coding regions
25 of the AT₄ receptor/IRAP gene. The oligonucleotides were 3' end labelled with ³³P-dATP using terminal d-transferase and purified on a P6 column. The mouse sections were hybridized with 1 x 10⁶ cpm of labelled oligonucleotide in a 100ml total volume of 50% formamide, 4xSSC, 1xDenhardt's
30 solution, 2% sarcosyl, 20mM sodium phosphate buffer (pH7.0), 10% dextran sulphate, 50µg/ml herring sperm DNA and 0.2mM dithiothreitol. After a 16h hybridization period at 42°C, the sections were washed four times in 1xSSC at 55°C, rinsed in distilled water and dehydrated
35 through increasing alcohol. The sections were then exposed to X-ray film. The results are shown in Figure 8.

The highest level of AT₄ receptor/IRAP protein was found in developing bone. High levels of the protein were also detected in the heart, kidney, thymus, pituitary and the subventricular zone of the brain (Figure 8).

5 These results suggest that the AT₄ receptor/IRAP system might play an important role in the development of these tissues. The distribution of AT₄ receptor/IRAP in the foetal and neonatal mice closely resemble the distribution of GLUT4 mRNA (Venucci et al., 1999). This suggests that
10 AT₄ receptor/IRAP may have an autoregulatory role in the transport of glucose into cells during development, as is the case in adults.

15 EXAMPLE 27 LVV-HAEMORPHIN 7 AND ANG IV INDUCTION OF DNA SYNTHESIS IN NEURONAL CELL LINES

Ang IV has been reported to induce DNA synthesis in rabbit and rat cardiac fibroblasts and in rat anterior pituitary cells. Moreover, in bovine coronary venular endothelial cells, Ang IV enhanced ³H-thymidine
20 incorporation in response to stimulation with basic fibroblast growth factor, although Ang IV alone was a very weak stimulant.

The effect of LVV-haemorphin 7 and Ang IV on cellular proliferation was investigated in cells of the
25 neuronally-derived immortalised cell lines NG108-15 and SK-N-MC, as appreciable amounts of AT₄ receptors/IRAP were shown to be present in these cell lines. NG108-15 is a rat-mouse hybrid cell line consisting of both neurones and glia, whereas the SKN-MC cells are neuroepithelial in
30 origin. SK-N-MC cells are known to display cholinergic properties, such as the expression of choline acetyltransferase and muscarinic receptors.

NG108-15 and SK-N-MC cells were cultured in DMEM supplemented with 10% heat-inactivated FCS, 2% HAT
35 supplement, and 100U/ml penicillin/streptomycin. Cells were routinely used between passages 20 and 30.

Cells were plated at a density of 3x10⁴

cells/well in 24 well Falcon plates that had been coated with 0.1mg/ml poly-L-lysine hydrobromide. The cells were allowed to adhere overnight, and then serum deprived for 48h before being treated with LVVYPWTQRF (SEQ ID NO:2) and/or Ang IV (10^{-10} to 10^{-6} M) for 24h in medium with or without serum. Five hours before the end of the experiment 1µCi of ^3H -thymidine was added to each well. At the completion of the incubation, cells were washed twice with 1ml of PBS at 4°C, followed by 5% trichloroacetic acid at 4°C for 30min, which was aspirated before solubilizing the cells in 1ml 0.25 M NaOH and 0.1% SDS for 30min at 37°C. Samples were then collected into 5ml of Ready Value scintillation fluid and ^3H measured in a liquid scintillation counter. The results are summarised in Figure 21.

In the NG108-15 cells, LVVYPWTQRF (SEQ ID NO:2) at concentrations of 10^{-10} to 10^{-8} M stimulated ^3H -thymidine incorporation in a dose-dependent manner to between 140 to 170% of control levels ($p < 0.05$). In contrast, at concentrations of 10^{-10} to 10^{-6} M, Ang IV did not stimulate DNA synthesis in these cells. The ability of LVVYPWTQRF (SEQ ID NO:2) to increase ^3H -thymidine incorporation was completely abolished by concomitant presence of 10^{-6} M Ang IV, in which situation the result was not significantly different from control ($p = 0.337$). Ang IV dose-dependently inhibited the effect of 10^{-6} M LVVYPWTQRF (SEQ ID NO:2) in stimulating ^3H -thymidine incorporation. At 10^{-10} M, Ang IV was inactive, whereas increasing concentrations of Ang IV from 10^{-9} to 10^{-7} M progressively inhibited ^3H -thymidine incorporation.

Treatment of SK-N-MC cells with increasing concentrations of Ang IV (10^{-10} - 10^{-7} M) significantly increased [^3H]-thymidine uptake, by up to 72% above control levels. LVVYPWTQRF (SEQ ID NO:2) also significantly stimulated [^3H]-thymidine uptake at 10^{-10} - 10^{-6} M, by up to 81% above control levels, with maximal effects observed at 10^{-9} to 10^{-7} M LVVYPWTQRF (SEQ ID NO:2) ($P < 0.001$). This

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effect was different to that observed in NG108-15 cells, where the stimulatory effect of LVVYPWTQRF (SEQ ID NO:2) was attenuated by Ang IV, with Ang IV alone not having any effect on DNA synthesis. In SK-N-MC cells, both peptides
5 stimulated DNA synthesis in a dose-dependent manner.

The lack of effect of Ang IV on ³H-thymidine incorporation in NG108-15 cells was not due to the degradation of the peptide. In a parallel study, using conditions similar to that of the DNA synthesis
10 experiments, the integrity of the peptide was determined by HPLC. Ang IV did undergo some degradation, but this was insufficient to explain its lack of activity.

EXAMPLE 28 LVVYPWTQRF (SEQ ID NO:2) AND ANG IV INHIBITS
15 NEURITE OUTGROWTH IN CHICK SYMPATHETIC
 NEURONES

The wide distribution of the AT₄ receptors/IRAP in the brain suggests important roles for this enzyme in the brain in the processing of several neuropeptides.
20 Moreover, the distribution of AT₄ receptors/IRAP closely correlated with the distribution of cholinergic neurones and their processes. Acetylcholine, the neurotransmitter in cholinergic neurones, is known to inhibit neurite outgrowth in embryonic chick ciliary ganglion cells. We
25 have examined whether either of the AT₄ receptor ligands, Ang IV and LVVYPWTQRF (SEQ ID NO:2), had a trophic effect in the central nervous system, and whether the effect paralleled that of acetylcholine. We examined whether either peptide exerted any effect on neurite outgrowth
30 from cultured chick sympathetic neurones.

Sympathetic ganglia from embryonic day 11 chicks were dissociated with trypsin/versene and were cultured in 24 well plates in DMEM and Ham's 12 medium which contained 1% insulin-transferrin-selenium-X growth supplement, 100 mM
35 putrescine, 1.67mg/ml prostaglandin F2 α , 6.67ng/ml progesterone, and 5ng/ml nerve growth factor. Neurones were allowed to adhere to the wells (approximately 2h)

before being treated with the peptides. Dose response curves were performed over the concentration range 10^{-11} to 10^{-5} M. Culture dishes were coated with 0.1mg/ml poly-L-lysine and washed with PBS before receiving another
5 coating of laminin (10µg/ml).

At the conclusion of the incubation, the culture medium was removed from the wells, the neurones fixed with 2.5% glutaraldehyde in phosphate-buffered saline for 20min, and examined under phase contrast microscope
10 attached to an MD30 Plus Image Analysis software system. The length of neurites (longer than 50µm) of every neurone examined was measured. A minimum of 40 neurite measurements was taken per treatment group, and each dose of peptide was repeated at least three times. At the
15 conclusion of the experiment, the viability of the cells was confirmed by exclusion of 0.1% aniline blue. The results are shown in Figure 22.

In cultures of embryonic chick sympathetic neurones, Ang IV and LVVYPWTQRF (SEQ ID NO:2) inhibited
20 neurite outgrowth in a dose dependent manner, with maximal effect achieved at concentrations between 10^{-9} to 10^{-8} M. The maximal extent of inhibition of neurite outgrowth was approximately 30%, which was comparable to the effect achieved by acetylcholine. These results suggest a role
25 for AT₄ receptors/IRAP in remodelling of neurones.

EXAMPLE 28 ROLE OF AT₄ RECEPTOR/IRAP IN BONE DEVELOPMENT

As described in Example 26, we also found a high concentration of AT₄ receptor/IRAP in the developing bone
30 of E13, E15, E17 mouse embryos. IGF-I receptors have been shown to co-localize with GLUT4 in chondroblastic and hypertrophic cells of mouse bone growth centres. Moreover, in the streptozotocin-induced diabetic mouse model, there are severe histological changes in the
35 mandibular condyles and humeral growth plate which are associated with decreases in GLUT4 and IGF-I and insulin receptors. We therefore consider that AT₄ receptor/IRAP

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co-localizes with GLUT4, and plays an important role in the regulation of IGF-I in regulating early bone growth.

EXAMPLE 30 EFFECT OF AT₄ RECEPTOR/IRAP LIGANDS ON BRAIN
5 DAMAGE

Activated astrocytes which are glial fibrillary acidic protein positive are involved in neuronal tissue remodelling after damage (Bovolenta et al 1992). Injury-evoked plasticity is similar to events observed in the
10 developing central nervous system (Schwartz 1992). IGF-I is a growth factor which is not only important in normal growth and development; there is strong evidence to suggest that it has an important role in neurodegenerative conditions in adult. Injury to the brain results in an
15 up-regulation of IGFs, their receptors and binding proteins. Application of exogenous IGF-I protects the brain from hypoxic and ischemic damage. In the light of our findings of high levels of AT₄ receptor/IRAP expression in the developing brain, as shown in Figure 23, and an
20 induction of AT₄ receptor/IRAP expression in activated astrocytes in mechanically-damaged spinal cord, as shown in Figure 24, we hypothesise that modulators of AT₄ receptor/IRAP activity will have a neuroprotective role in different animal models of damage.

25 To test this hypothesis, male Sprague-Dawley rats are anaesthetized with isofluorane in O₂ and indwelling intracerebroventricular cannulae are implanted. The cannula are connected to Alzet minipumps containing artificial cerebrospinal fluid which are implanted
30 subcutaneously in the dorsal neck region. Seven days later, the rats are re-anaesthetised with halothane in O₂, and focal cerebral ischemia induced by permanent occlusion of the left, middle cerebral artery proximal to the lenticulostriate branch, by electrocoagulation. During
35 the surgery, the Alzet minipumps containing artificial cerebrospinal fluid are replaced with ones containing either:

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- 1) Artificial cerebrospinal fluid; or
- 2) 10 or 100pmol/h Nle-Ang IV; or
- 3) 10 or 100pmol/h LVVYPWTQRF (SEQ ID NO:2).

Two or seven days after induction of the cerebral
5 ischemia, the animals are killed, the brains removed, and
50µm sections are taken and stained with 2% tri-phenyl-
tetrazolium chloride. The volumes of the infarcts are
determined on the sections from peptide-treated animals,
and compared to those in artificial cerebrospinal fluid
10 controls.

EXAMPLE 31 ROLE OF IRAP/AT₄ RECEPTOR IN BLOOD VESSELS

The vascularisation of tissues during development
occurs by the process of angiogenesis, in which
15 endothelial cells from existing capillaries form new
capillaries by sprouting or by splitting from their tissue
of origin. Little angiogenesis occurs in adult tissues,
except during wound healing, during the female
reproductive cycle and in certain pathological states such
20 as cancer. Pathological angiogenesis involves the
persistent proliferation of endothelial cells, as occurs
in proliferative retinopathy, rheumatoid arthritis,
psoriasis and tumour growth. Angiogenesis is also
essential to the growth of solid tumours.

25 The observed occurrence of AT₄ receptor/IRAP in
developing blood vessels near the site of a wound,
illustrated in Figure 24, suggests that AT₄ receptor/IRAP
has a role in angiogenesis during wound healing, and
possibly in the pathological conditions mentioned above.
30 If this is so, the potential exists to use modulators of
IRAP/AT₄ to interfere with angiogenesis and thereby limit
tumour invasion, and/or to treat other conditions in which
pathogenic angiogenesis is a feature. Peptidases are known
to have many roles in angiogenesis. Degradation of
35 extracellular matrix (ECM) by peptidases allows the
infiltration of endothelial cells from existing blood
vessels.

ECM degradation is a tightly controlled process, and peptidases also play roles in its regulation. For instance, ECM-degrading proteinases mobilise growth factors such as fibroblast growth factor (FGF) from the ECM, which stimulates expression of collagenase and plasminogen activator by microvascular endothelial cells. ECM-degrading proteinases also modulate activation of latent TGF- β 1 in the ECM, which counteracts the stimulatory effects of FGF on vascular endothelial cells.

A number of experimental systems are used to test the role of AT₄ receptor/IRAP in angiogenesis. When endothelial cells are grown on a gel formed of reconstituted basement membrane proteins (Matrigel), they aggregate and organise into tubule-like structures (Kubota et al., 1988). The ability of peptides that bind to AT₄ receptor/IRAP (e.g. Ang IV, LVVYPWTQRF (SEQ ID NO:2), KYLPGPLQ (SEQ ID NO:3) and divalinal-Ang IV) to modulate this process is assessed by culturing human umbilical vein endothelial (HUVE) cells or human dermal microvascular cells on Matrigel, with or without pre-incubation of the cells with IRAP/ AT₄ receptor -binding peptide. Alternatively the peptide is incorporated into the gel matrix. The role of AT₄ receptor/IRAP in angiogenesis is further assessed by incorporating antibodies to AT₄ receptor/IRAP into the matrigel before application of HUVE cells or HDME cells.

The role of IRAP/AT₄ in tumour angiogenesis is initially investigated using receptor autoradiography with [125I]-Nle-Ang IV to examine the distribution of IRAP/AT₄ in the microvasculature in sections of human tumour tissue. Next, the ability of IRAP/AT₄ ligands to modulate tumour angiogenesis is assessed in an *in vivo* model in which C3L5 cells, a highly metastatic murine mammary adenocarcinoma cell line, are suspended in growth factor-reduced Matrigel and implanted subcutaneously into mice. Growth factor-reduced Matrigel alone does not stimulate angiogenesis (Jadeski & Lala, 1999), but angiogenesis

occurs in implants containing tumour cells. IRAP/AT₄ ligands are administered via osmotic minipumps over 14 days, after which their effects on angiogenesis within the implants are assessed.

5

EXAMPLE 32 UPREGULATION OF AT₄ RECEPTOR/IRAP IN
ATHEROSCLEROTIC BLOOD VESSELS

Ang IV binds to AT₄ receptors in blood vessels to induce vasodilatation and proliferation of cultured bovine endothelial cells. Topical application of Ang IV caused vasodilatation of pial arterioles, and infusion of Ang IV into the rat middle cerebral artery and renal artery significantly increased blood flow. Ang IV significantly but modestly increased DNA synthesis in cultured bovine endothelial cells, and synergistically enhanced basic fibroblast growth factor-stimulated cellular proliferation.

Twelve week old male New Zealand White rabbits were anaesthetised with propofol (10 mg/kg, intravenously) followed by halothane, administered via an endo-tracheal tube. A 2F Fogarty embolectomy catheter was inserted through an arteriotomy of a branch of the external right carotid artery and extended to the aortic arch. The catheter was inflated and withdrawn to the bifurcation of the common carotid artery in order to de-endothelialize the vessel. This was repeated three times to ensure complete de-endothelialization. The uninjured left carotid arteries were used as control vessels. Three rabbits per group at 1 day and 1 week post surgery, and 4 rabbits per group at 4 weeks and 20 weeks post surgery, were killed with a lethal dose of pentobarbitone and left and right carotid arteries were removed and rapidly frozen in isopentane cooled to -40°C.

Carotid arteries were sectioned at 20µm thickness. Sections were preincubated in an isotonic buffer, 50mM Tris-HCl buffer, pH 7.4, 150mM NaCl and 5mM EDTA for 30min at 22°C and then incubated in 50mM Tris-HCl,

pH 7.4, 150mM NaCl, 5mM EDTA, 100µM phenylmethyl-sulfonyl fluoride (Sigma, Mo., U.S.A.), 20µM bestatin (Peninsula Laboratories, Ca., U.S.A.) and 0.1% bovine serum albumin, containing approximately 200pM [¹²⁵I] Ang IV, for 2h at
5 22°C. Non-specific binding was determined in the presence of 1µM Ang IV in adjacent sections. Following the incubation, the sections were given 3 x 2min washes in the isotonic buffer, at 4°C, air dried, and exposed to X-ray film for 3.5 weeks, together with a set of radioactive
10 standards. After exposure, the sections were counterstained with haematoxylin and eosin for histological examination.

Images were quantified on an MCID image analysis system (Image Research, Ontario Canada), in which the
15 pixel densities on the X-ray films were converted to radioactive units (dpm/mm²) using the radioactive standards. The vessel wall thickness was measured using the MCID system. The results are shown in Figures 25 and 26.

20 In normal rabbit carotid arteries, AT₄ receptors were detected in vascular smooth muscle cells and in the vasa vasorum of the adventitia, as shown in Figure 25, left-hand panel. Very low receptor levels were observed in the endothelial cells.

25 Within the first week following endothelial denudation of the carotid artery by balloon catheter, AT₄ receptor/IRAP in the media/neointima increased to approximately 150% of control tissue, as shown in Figures 26A and 26B. AT₄ receptor/IRAP further increased in the
30 media/neointima and re-endothelialized cell layer to 223% at 20 weeks after injury (Figures 26C and 26D).

In view of the known trophic effects of Ang IV, the elevated expression of IRAP/AT₄ receptors in both the neointima and media of arteries following balloon injury
35 to the endothelium, suggests a role for the IRAP/AT₄ receptor in the adaptive response and remodelling of the vascular wall following damage.

EXAMPLE 33 Elucidation of factors which translocate AT₄ receptor/IRAP to the cell surface in neuronal cells

5 In insulin-responsive cells in the basal state, a major proportion of AT₄ receptor/IRAP and GLUT4 is co-localised inside the cell in specialised post-endosomal vesicles, which are distinct from constitutively recycling endosomes, and also in the trans-Golgi network. Both GLUT4
10 and AT₄ receptor/IRAP rapidly translocate to the plasma membrane in response to insulin stimulation; GLUT4 to mediate insulin-stimulated glucose uptake (Garza et al, 2000).

 This process involves the formation of vesicles
15 containing both GLUT4 and AT₄ receptor/IRAP, which translocate to the cell surface, and fuse with the plasma membrane by a mechanism which in part parallels synaptic vesicle trafficking and docking in the regulation of neurotransmitter release in the brain (Cheatham 2000). The
20 molecular mechanism/signalling cascade regulating the trafficking of GLUT4/ AT₄ receptor/IRAP vesicles has not been fully elucidated. In addition to insulin, osmotic shock, guanosine 5'-O-(3-thiotriphosphate) (GTPγS) and adrenergic stimulation also result in the translocation of
25 AT₄ receptor/IRAP and GLUT4 to the cell surface in adipocytes and/or skeletal muscle cells. In human vascular endothelial cells, activation of the oxytocin receptor by oxytocin, a substrate for AT₄ receptor/IRAP, was demonstrated to translocate IRAP to the cell surface
30 (Nakamura et al, 2000).

 We have established stably transfected cell lines of both SK-N-MC and SN56 cells expressing AT₄ receptor/IRAP with a c-Myc tag at the extracellular carboxy terminal. These cell lines enable us to rapidly and quantitatively
35 determine the factors or compounds which result in the translocation of IRAP to the cell surface. Serum-deprived cells are incubated with different candidates, such as

insulin, IGF-I, neurotensin, or AT₄ receptor/IRAP substrates, or under depolarizing conditions, and the level of AT₄ receptor/IRAP at the plasma membrane measured after 30 min using an in-house ELISA assay. After positive
5 factors have been identified, time-course studies are performed.

The results using this system are verified by performing subcellular fractionation using sucrose gradient centrifugation, followed by Western Blot analysis
10 of AT₄ receptor/IRAP in stimulated and unstimulated cells. The first set of experiments measures endogenous AT₄ receptor/IRAP in untransfected cells, to verify that the results obtained are not an artefact of the transfection system used. In the second series of experiments we
15 determine the subcellular vesicles which are recruited to the plasma membrane following stimulation. Light density microsomes from stimulated and non-stimulated cells are immunoadsorbed with antibodies to the different vesicle marker proteins, and the level of AT₄ receptor/IRAP for
20 each vesicle type determined by Western Blot analysis.

We have demonstrated that serum stimulates the translocation of IRAP to the cell surface in both SK-N-MC and SN56 cells. We are identifying the factors, compounds or conditions which will translocate IRAP to the cell
25 surface.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments
30 and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

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35 reference.

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CLAIMS

1. A method for modulating the expression,
production, or activity of AT₄ receptor/IRAP in a subject,
5 comprising the step of administering an effective amount
of a compound which modulates AT₄ receptor/IRAP expression,
production or activity, in which the compound is
- (a) a ligand of AT₄ receptor/IRAP,
 - (b) an oligonucleotide molecule which is antisense to
10 a nucleic acid encoding AT₄ receptor/IRAP, or
 - (c) a compound which is able to modulate the cellular
localization of AT₄ receptor/IRAP
- to the subject.
2. A method of screening for compounds which have
15 the ability to modulate the activity of AT₄ receptor/IRAP,
comprising the steps of
- (a) assessing the ability of a candidate compound to
bind to AT₄ receptor/IRAP, and one or more of
 - (b) assessing the ability of the compound to modulate
20 the enzymatic activity of AT₄ receptor/IRAP;
 - (c) assessing the effect of the compound on the
interaction between AT₄ receptor/IRAP and a
protein which interacts with AT₄ receptor/IRAP;
 - (d) assessing the ability of the candidate compound
25 to modulate one or more signalling or
intracellular pathways activated by binding of
the candidate compound to AT₄ receptor/IRAP;
 - (e) assessing the ability of the candidate compound
to modulate an activity of AT₄ receptor/IRAP;
 - 30 (f) assessing the ability of the candidate compound
to translocate AT₄ receptor/IRAP to the cell
surface.
3. A method according to claim 2, comprising the
steps of assessing the effect of the candidate compound on
- 35 a) binding to AT₄ receptor/IRAP or to a protein

- which interacts with AT₄ receptor/IRAP;
- b) altering the ability of a protein to interact with AT₄ receptor/IRAP; and
- c) modifying the enzymatic or signalling activities of AT₄ receptor/IRAP.
- 5 4. A method according to claim 2 for identifying compounds which translocate AT₄ receptor/IRAP to the cell surface, comprising the steps of
- (a) exposing cells of a stably transfected cell line expressing AT₄ receptor/IRAP to a candidate compound, and
- 10 (b) assessing the amount of AT₄ receptor/IRAP expressed on the cell surface.
5. A method according to any one of claims 2 to 4, in which the compound is an intracellular or extracellular
- 15 protein.
6. A method according to claim 2 or claim 3, in which the protein which interacts with AT₄ receptor/IRAP is tankyrase.
7. A method for modulating the expression, production, or activity of AT₄ receptor/IRAP, comprising the step of administering an effective amount of a compound which alters AT₄ receptor/IRAP expression, production or activity to a subject in need of such treatment, in which the compound is a specific, high
- 20 affinity ligand of AT₄ receptor/IRAP which modulates AT₄ receptor/IRAP levels or AT₄ receptor/IRAP activity.
8. A method of modulating the *in vivo* concentration of a neuropeptide and/or a polypeptide, comprising the step of administering an effective amount of a compound
- 30 which can modulate an effect mediated by AT₄ receptor/IRAP to a subject in need of such treatment.
9. A method according to claim 8, in which the neuropeptide is selected from the group consisting of arginine-vasopressin, oxytocin, somatostatin, angiotensin
- 35 III, Lys-bradykinin, neurotensin, met-enkephalin, dynorphin A, neurokinin A, neuromedin B, and cholecystokinin 8.

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10. A method according to claim 8, in which the polypeptide is selected from the group consisting of insulin, insulin-like growth factor I and insulin-like growth factor II.
- 5 11. A method of prevention or treatment of a disease or condition associated with altered activity of AT₄ receptor/IRAP, comprising the step of administering an effective amount of a compound which has the ability to modulate an effect mediated by AT₄ receptor/IRAP to a
- 10 subject in need of such treatment.
12. A method for the prevention or treatment of a disease or condition by altering the activity of AT₄ receptor/IRAP, comprising the step of administration of an effective amount of a compound which has the ability to
- 15 modulate an effect mediated by AT₄ receptor/IRAP to a subject in need of such treatment.
13. A method according to claim 11 or claim 12, in which the disease or condition is
- (a) a disorder of the central nervous system
- 20 (CNS) associated with dementia and/or memory loss;
- (b) a disorder of the CNS involving motor and sensory systems;
- (c) a disorder of the CNS resulting from trauma or stroke;
- 25 (d) a disorder of the cardiovascular system;
- (e) a disorder of development and/or growth;
- (f) a disorder of glucose and fat metabolism;
- (g) a disorder of the reproductive tract;
- (h) a disorder associated with pregnancy; or
- 30 (i) a cancer.
14. A method according to claim 11 or claim 12, in which the modulator of an effect mediated by AT₄ receptor/IRAP is used for promotion of neuroregeneration and repair following CNS damage or injury, for promotion
- 35 of tissue regeneration and repair following damage or injury, for promotion of wound healing, for inhibition of angiogenesis, or for treatment of premature or delayed

labour.

15. A method according to any one of claims 11 to 14, in which the condition is selected from the group consisting of

- 5 (a) a neurodegenerative condition selected from the group consisting of motor neurone disease (amyotrophic lateral sclerosis), progressive spinal muscular atrophy, infantile muscular atrophy, Charcot-Marie-Tooth disease, Parkinson's Disease, Parkinson-Plus syndrome, Guamanian
10 Parkinsonian dementia complex, progressive bulbar atrophy, and Alzheimer's disease;
- (b) a neurodegenerative condition arising from ischaemia, hypoxia, neural injury, surgery, or exposure to neurotoxins;
- 15 (c) a peripheral sensory neuropathy resulting from exposure to drugs or toxins; and
- (d) a peripheral sensory neuropathy resulting from diabetes.

16. A method according to any one of claims 11 to 14
20 of preventing or treating a condition characterised by neuronal deficit or neuronal death, comprising the step of administering an effective amount of a compound which has the ability to modulate an effect mediated by AT₄ receptor/IRAP to a subject in need of such treatment.

25 17. A method according to claim 16 of stimulation of growth in an animal, comprising the step of administering an effective amount of a compound which can modulate an effect mediated by AT₄ receptor/IRAP to the animal.

18. A method according to claim 17, in which the
30 mammal is a farm animal.

19. A method according to claim 18, in which the farm mammal is selected from sheep, cattle, pigs, goats and poultry.

20. A method according to claim 10 of modulating the
35 *in vivo* uptake of glucose into cells or tissues, comprising the step of administering an effective amount of a compound which can modulate an effect mediated by AT₄

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receptor/IRAP to a subject in need of such treatment.

21. A method according to claim 14 of treating memory disorders and/or of reversing memory loss, comprising the step of administration of an effective amount of a
5 compound which has the ability to modulate an effect mediated by AT₄ receptor/IRAP to a subject in need of such treatment.

22. A method of enhancing memory and/or learning in a normal subject, comprising the step of administering an
10 effective amount of a compound which can modulate an effect mediated by AT₄ receptor/IRAP to a subject in need of such treatment.

23. A method according to any one of claims 2 to 22, in which the compound which modulates an effect mediated
15 by AT₄ receptor/IRAP is able to decrease or increase the enzymatic activity of AT₄ receptor/IRAP, thereby increasing the serum and tissue levels of biologically-active arginine-vasopressin, oxytocin, somatostatin, angiotensin III, Lys-bradykinin, neurotensin, met-enkephalin,
20 dynorphin A, neurokinin A, neuromedin B, and/or cholecystokinin 8.

24. A method of monitoring the activity of AT₄ receptor/IRAP, comprising the step of assessing the aminopeptidase activity of AT₄ receptor/IRAP in a
25 biological sample.

25. A method of diagnosing an AT₄ receptor/IRAP-related condition, comprising the step of measuring the level of activity of AT₄ receptor/IRAP in a subject suspected of having such a condition, and comparing the
30 level to that found in a normal individual, in which any difference between the levels is indicative of the condition.

26. A therapeutic or prophylactic agent for a disease or condition associated with altered activity of AT₄
35 receptor/IRAP, comprising
(a) a peptide which has an amino acid sequence selected from the group consisting of LVVYPWTQRF (SEQ ID

- NO:2), KYLPGPLQ (SEQ ID NO:3) and VYIH^HPF (SEQ ID NO:1), or an analogue or peptidomimetic thereof, which is able to modulate an effect mediated by AT₄ receptor/IRAP,
- (b) an inhibitor of leucine aminopeptidase activity
- 5 which has the ability to inhibit IRAP, and/or
- (c) an antisense AT₄ receptor/IRAP nucleic acid molecule or a fragment thereof which has the ability to inhibit expression of AT₄ receptor/IRAP, and optionally
- (d) a pharmaceutically acceptable carrier.
- 10 27. An agent according to claim 26, in which the inhibitor of leucine aminopeptidase activity is L-leucinethiol, L-leucinal, L-leucinol, or a derivative thereof which has the ability to inhibit IRAP.
28. An agent according to claim 27, in which the
- 15 peptide is KYLPGPLQ (SEQ ID NO:3), or an analogue or peptidomimetic thereof, which is able to modulate an effect mediated by AT₄ receptor/IRAP.
29. A method of modulating the activity of AT₄ receptor/IRAP, comprising the step of administering to a
- 20 patient in need thereof an effective amount of an antisense nucleic acid molecule directed against nucleic acid encoding AT₄ receptor/IRAP, or a fragment thereof which has the ability to inhibit expression of AT₄ receptor/IRAP.
- 25 30. A method according to claim 29, in which the antisense nucleic acid molecule directed against nucleic acid encoding AT₄ receptor/IRAP has a nucleotide sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID
- 30 NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27 and SEQ ID NO:28.
31. An oligonucleotide molecule which is antisense to a nucleic acid encoding AT₄ receptor/IRAP, which is
- 35 a) an oligonucleotide molecule which has a nucleotide sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ

ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27 and SEQ ID NO:28;

- 5 b) a fragment of a) which has the ability to inhibit expression of AT₄ receptor/IRAP;
- c) a nucleic acid molecule which has at least 75% sequence homology to a sequence in a) or b); or
- 10 d) a nucleic acid molecule which is capable of hybridizing to a sequence in a) or b) under stringent conditions;
- optionally operatively linked to a vector and/or present in a transfected host cell.
32. A construct for *in vivo* delivery of an effective
- 15 amount of antisense AT₄ receptor/IRAP to a subject, comprising:
- a) an oligonucleotide according to claim 30; and
- b) a vector comprising a control sequence,
- 20 wherein the control sequence is capable of controlling the expression of a nucleotide sequence of a), which in turns modulates the activity of AT₄ receptor/IRAP.
33. A construct according to claim 32, operatively linked to a vector and/or present in a
- 25 transfected host cell.
34. A composition comprising one or more agents according to claim 27, antisense oligonucleotides according to claim 30, or constructs according to claim 33, together with a pharmaceutically
- 30 acceptable carrier.

SEQUENCE LISTING

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<120> MODULATION OF AMINOPEPTIDASE ACTIVITY

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<141> 2001-08-02

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1 5 10

<210> 4

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<212> PRT

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28

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1/30

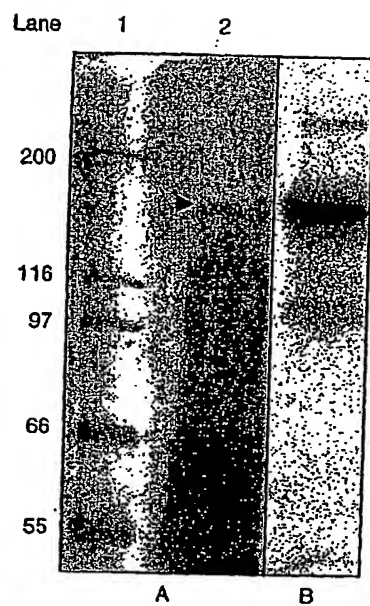
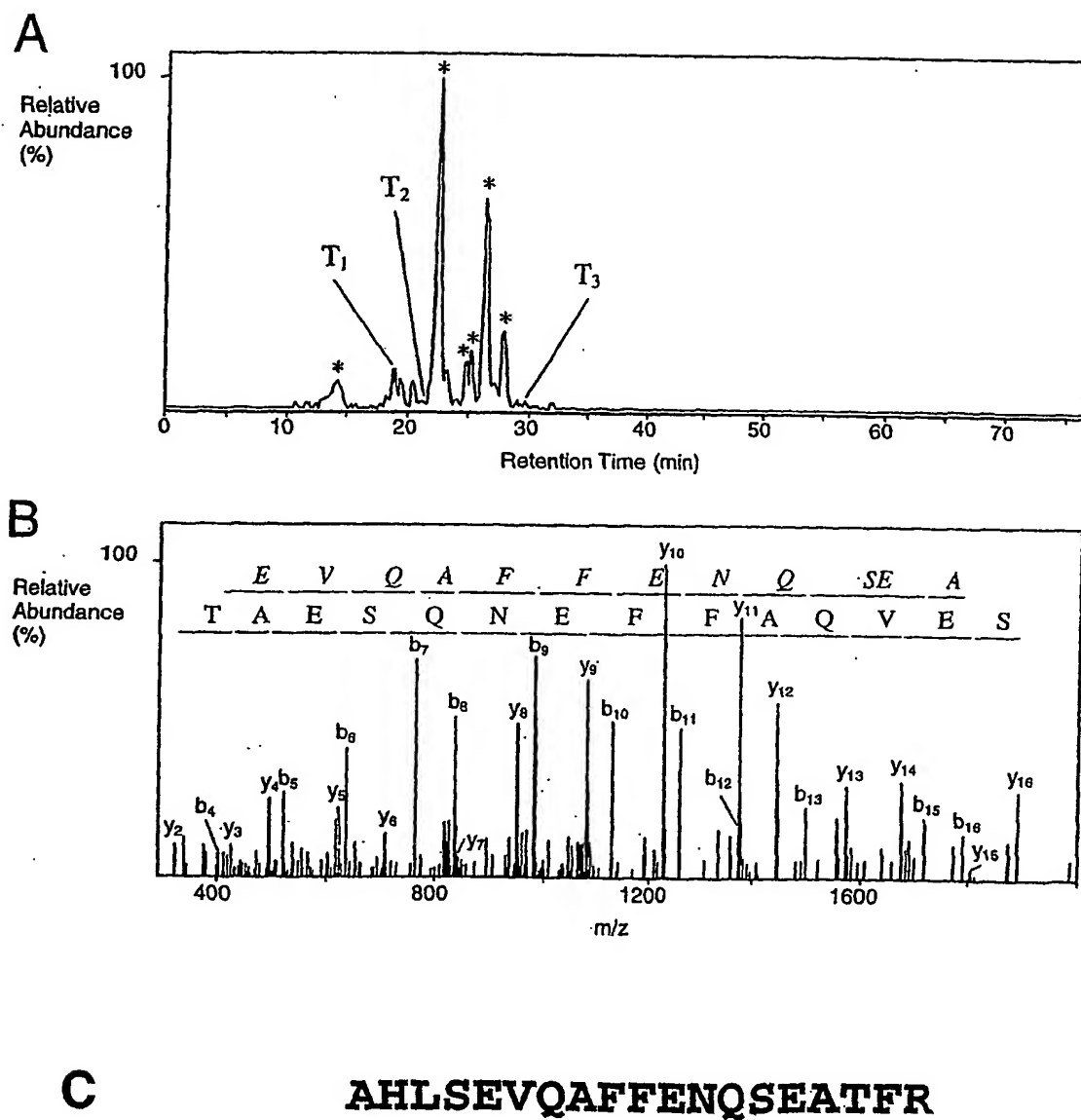
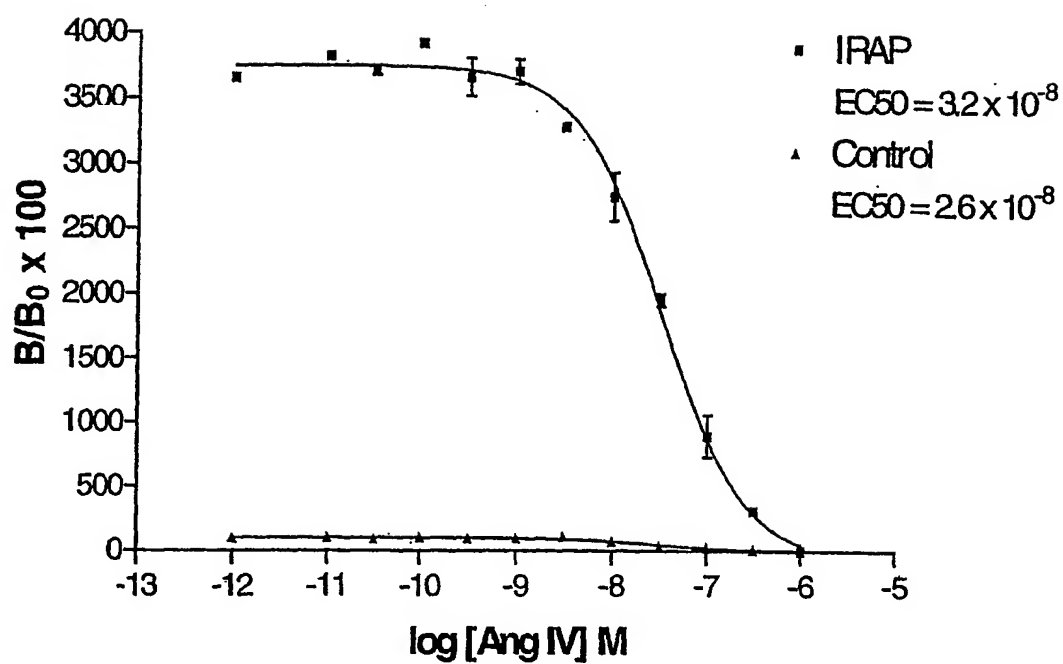


Figure 1

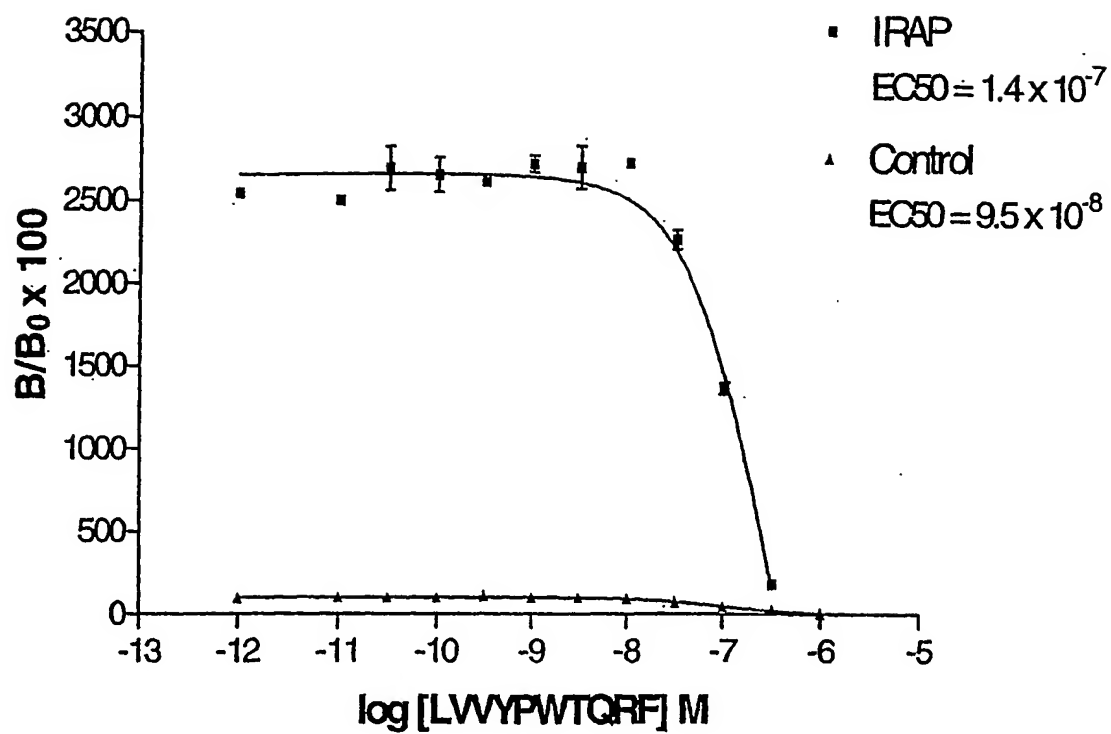
2/30

*Figure 2*

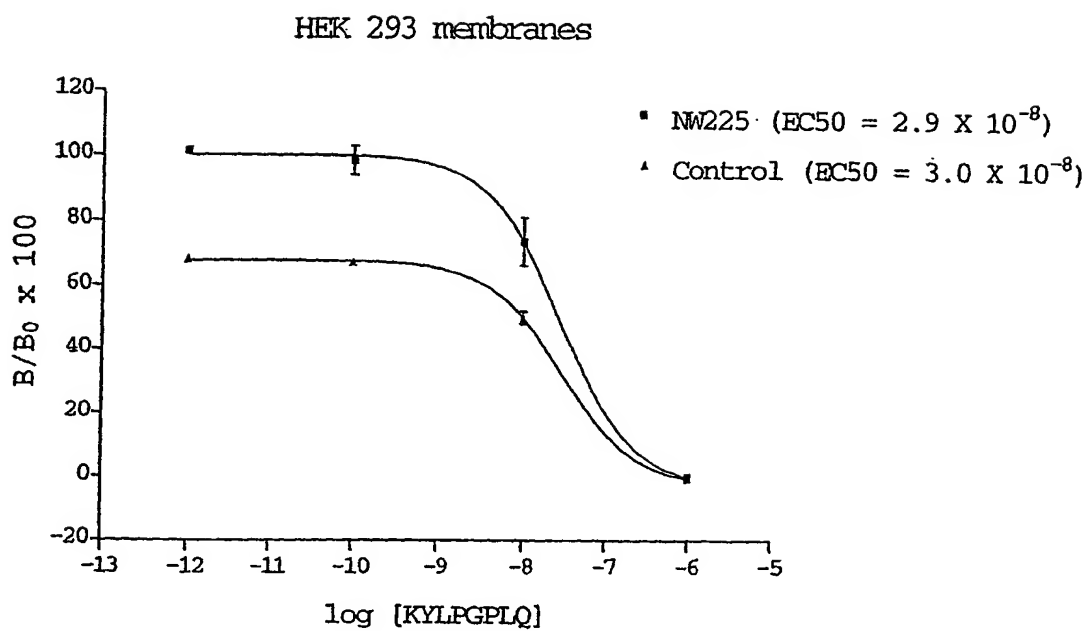
3/30

*Figure 3*

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*Figure 4*

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*Figure 5*

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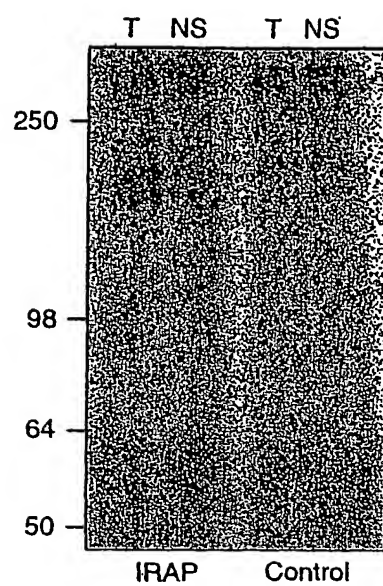
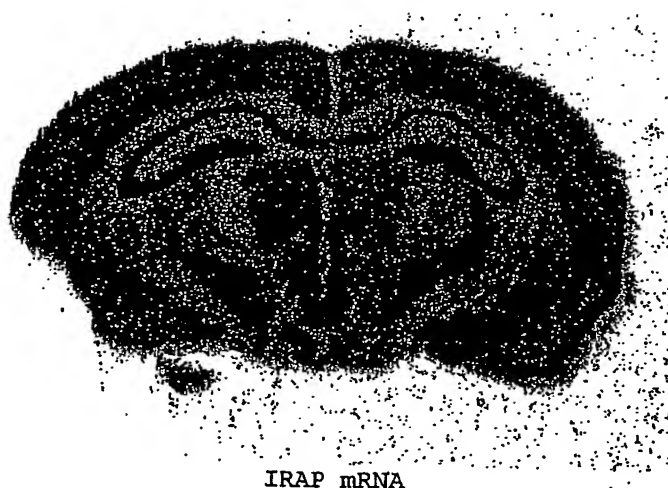


Figure 6

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AT4 receptor binding



IRAP mRNA

Figure 7

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AT4 receptor binding

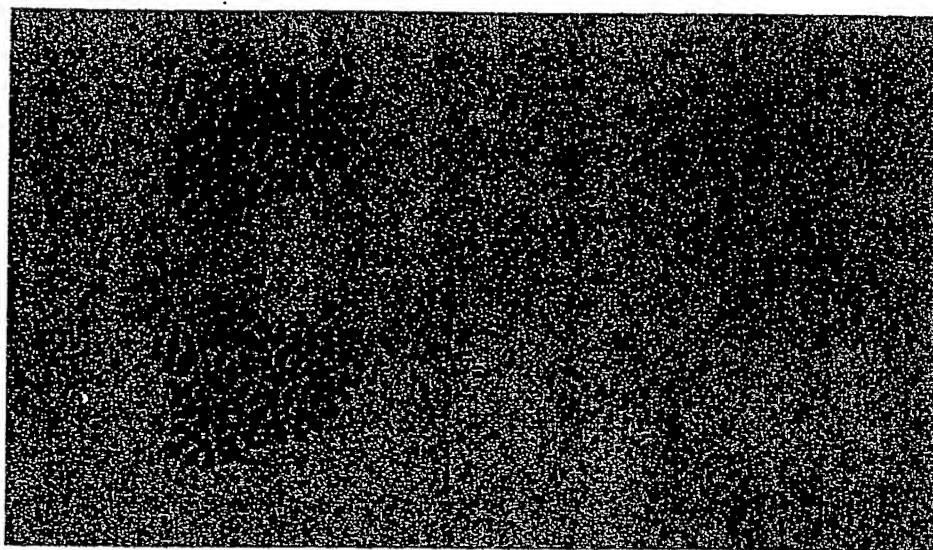


IRAP mRNA

Figure 8

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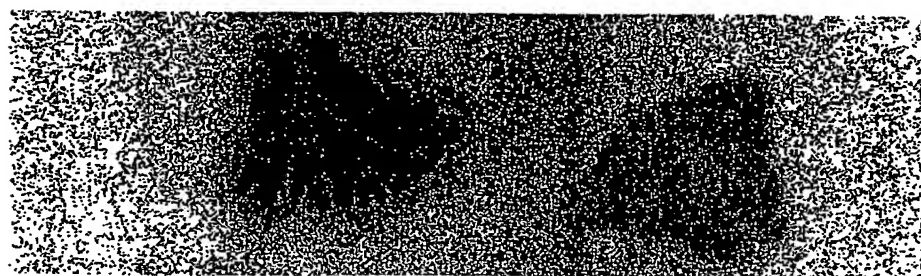
IRAP mRNA distribution in human cerebellum



antisense oligonucleotides

sense oligonucleotides

AT4 receptor distribution in human cerebellum



total binding

non-specific binding

Figure 9

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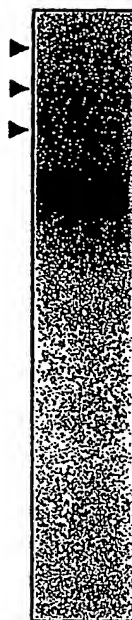


Figure 10

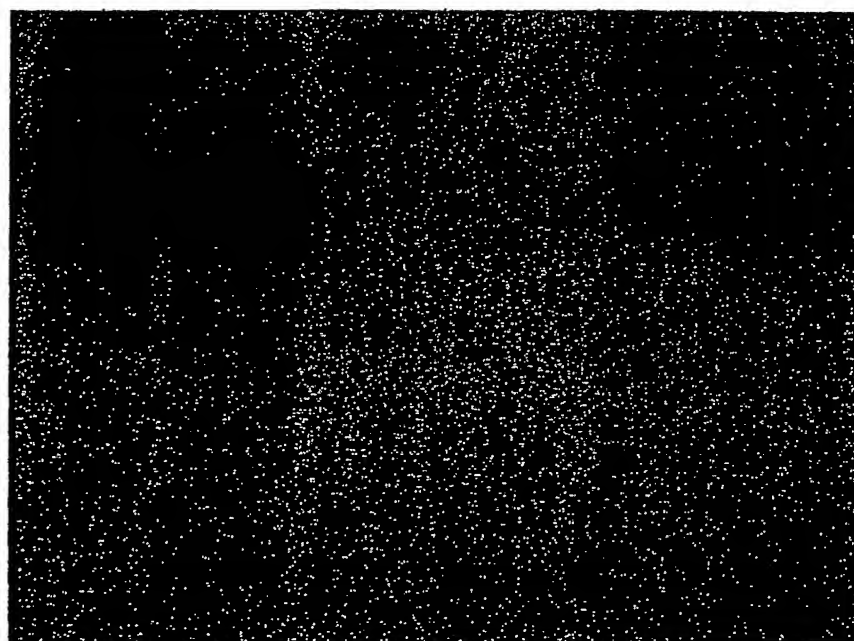
11/30

	1	2	3	4	5	6
DTT	-	+	-	+	-	+
10 μ M Ang IV	-	-	+	+	-	-
10 μ M LVV-H7	-	-	-	-	+	+

250 kDa ➤

180 kDa ➤

A.



1

2

3

4

5

6

250 kDa ➤

180 kDa ➤

B.

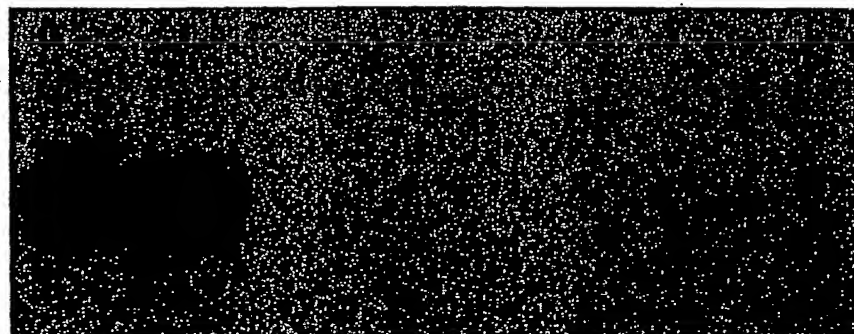
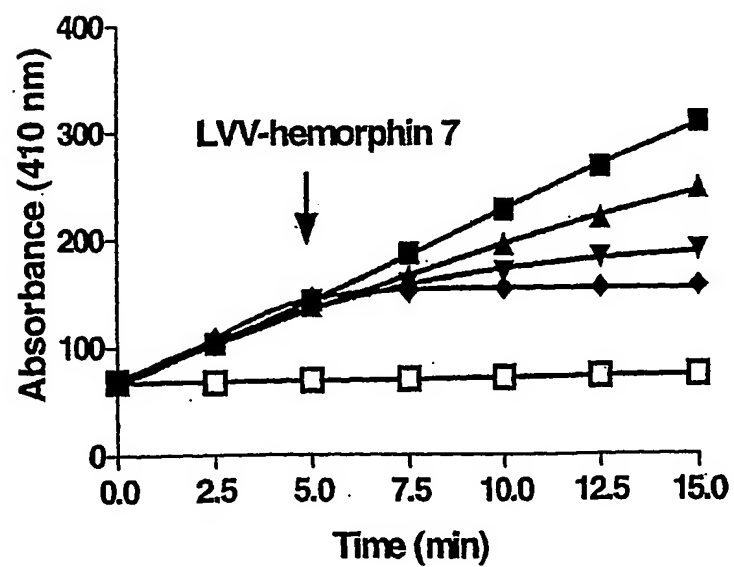
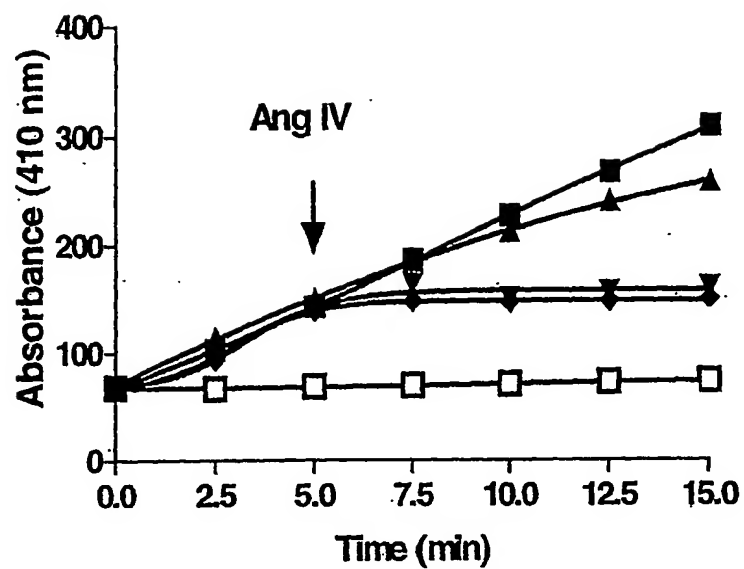
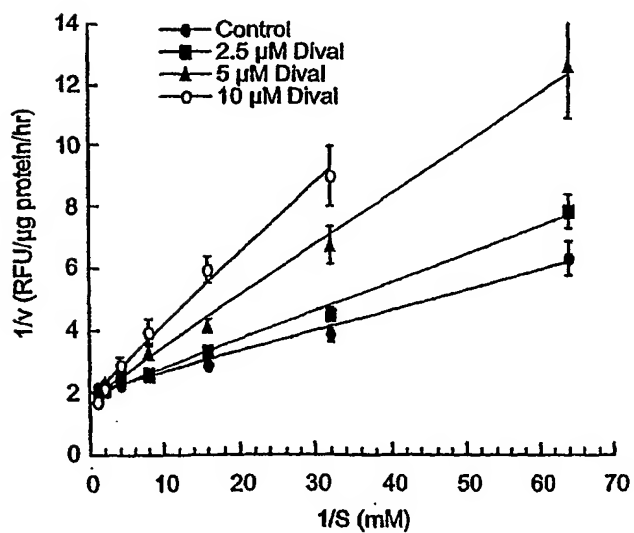
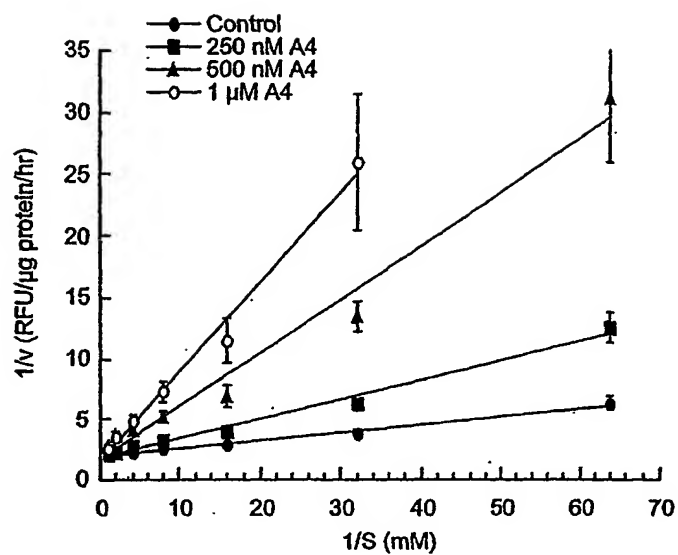


Figure 11

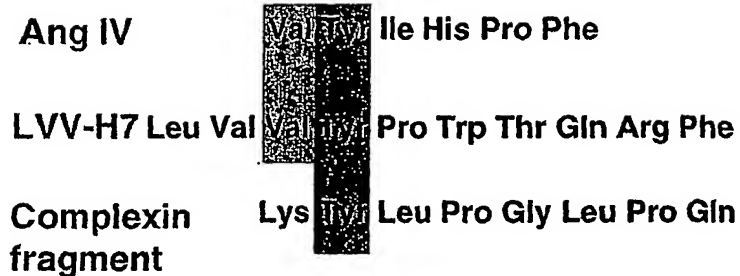
12/30

*Figure 12a*

13/30

*Figure 12b*

14/30



C- and N-terminal truncations of LVVYPWTQRF
- determination of amino acid residues
important for binding

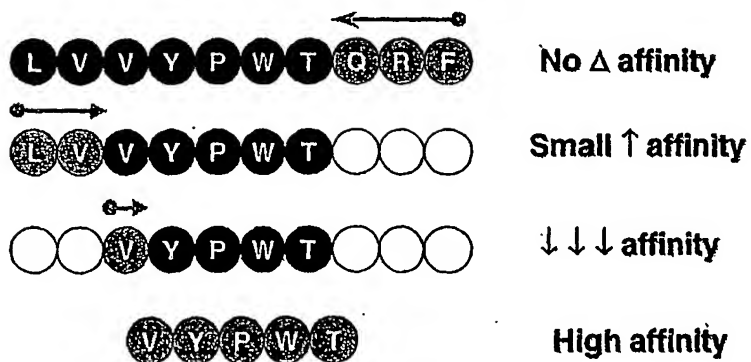
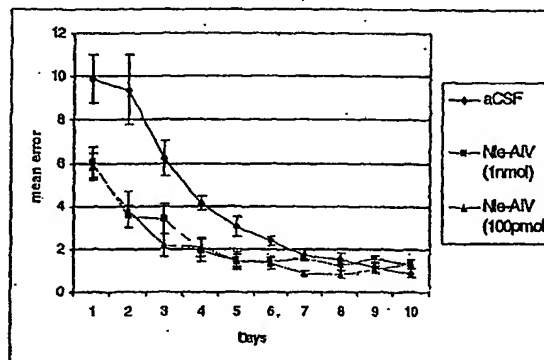


Figure 13

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**Effect of Nle-Ang IV on spatial memory in rats -
Barnes circular maze paradigm : Mean error**



**Effect of Nle-Ang IV on spatial memory in rats -
Barnes circular maze paradigm : days taken to
reach learner criteria**

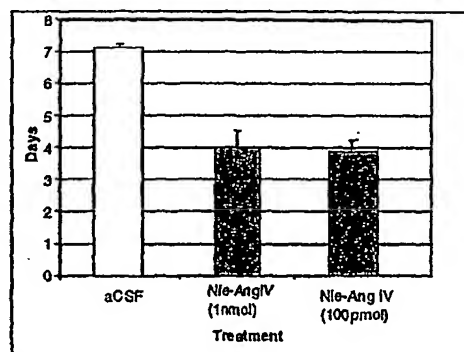
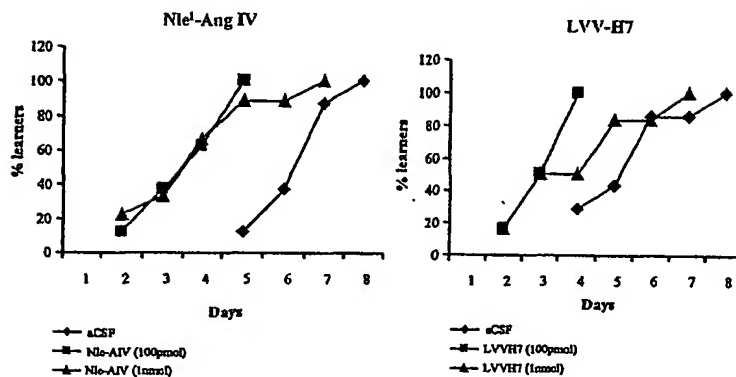


Figure 14a

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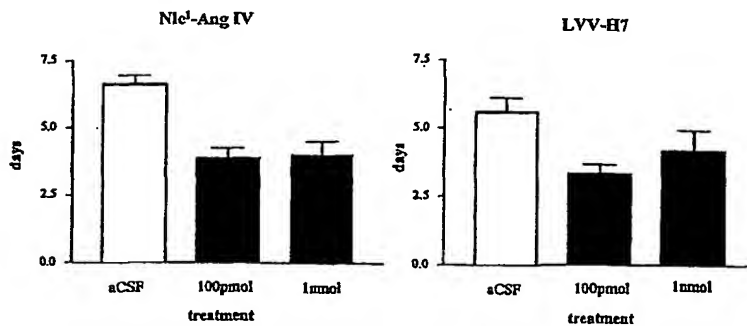
Effect of AT₄ ligands on spatial memory in rats - Barnes maze paradigm

Learner Curves



Effect of AT₄ ligands on spatial memory in rats - Barnes maze paradigm

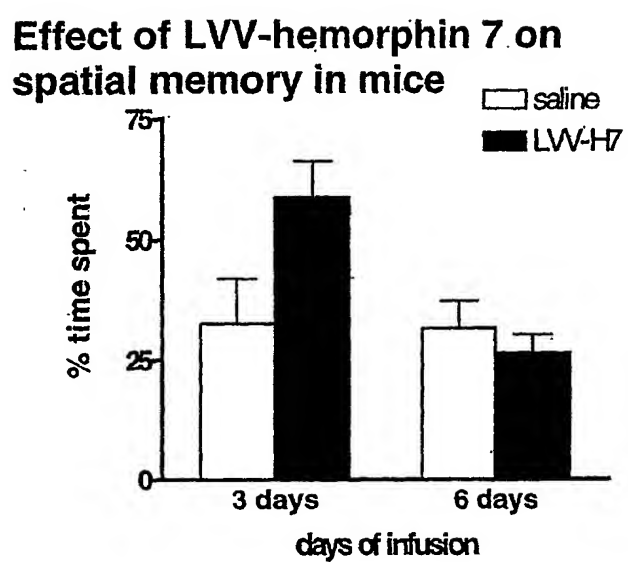
Mean days taken to achieve learner criterion



Learner criterion is achieved when the animal find and utilise the escape tunnel with mean of 2 or less errors over the three consecutive trials on any given day.

Figure 14b

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6h intertrial interval and at an infusion rate of 0.3pmol/h

Figure 15

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**Effect of LVV-hemorphin 7 on memory
: water maze paradigm**

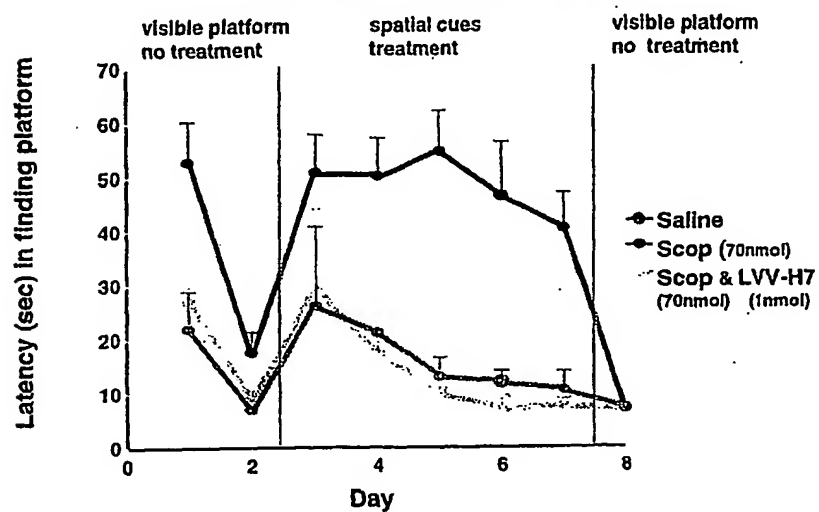
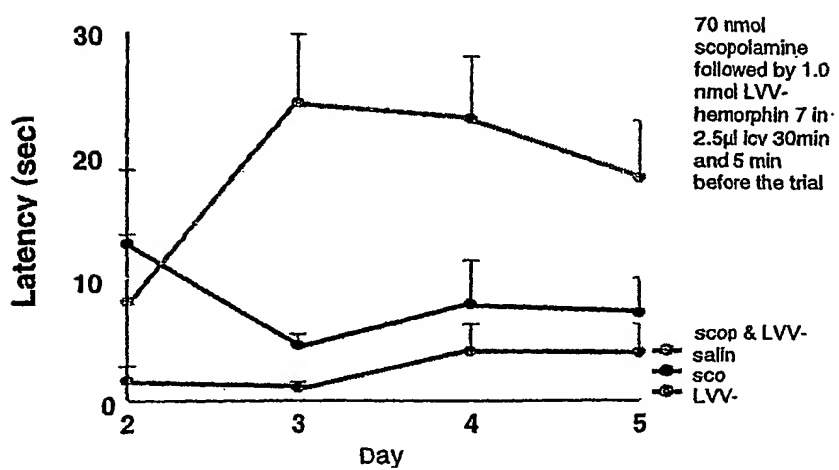


Figure 16

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Effect of LVV-hemorphin 7 on memory : passive avoidance

*Figure 17*

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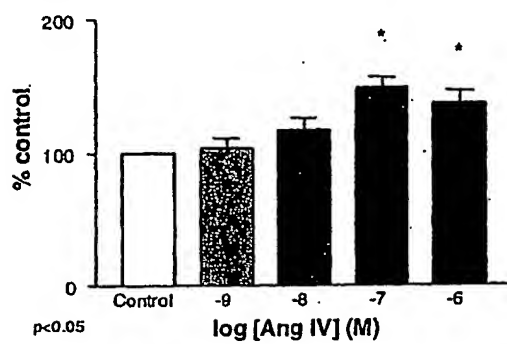
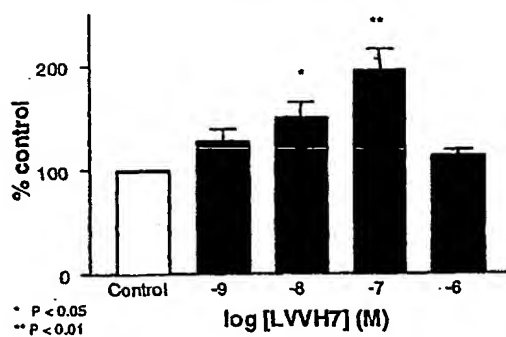
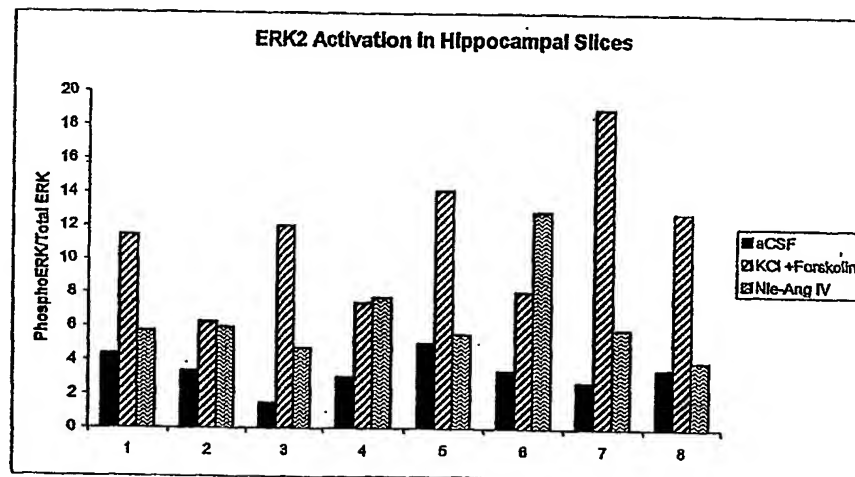
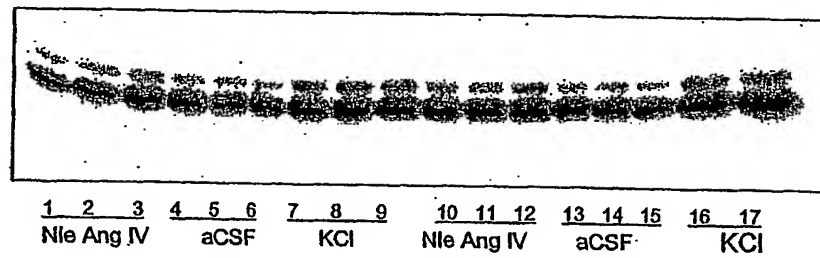
Effect of Ang IV on evoked ^3H -ACh releaseEffect of LVV-hemorphin-7 on evoked ^3H -ACh release

Figure 18

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*Figure 19a*

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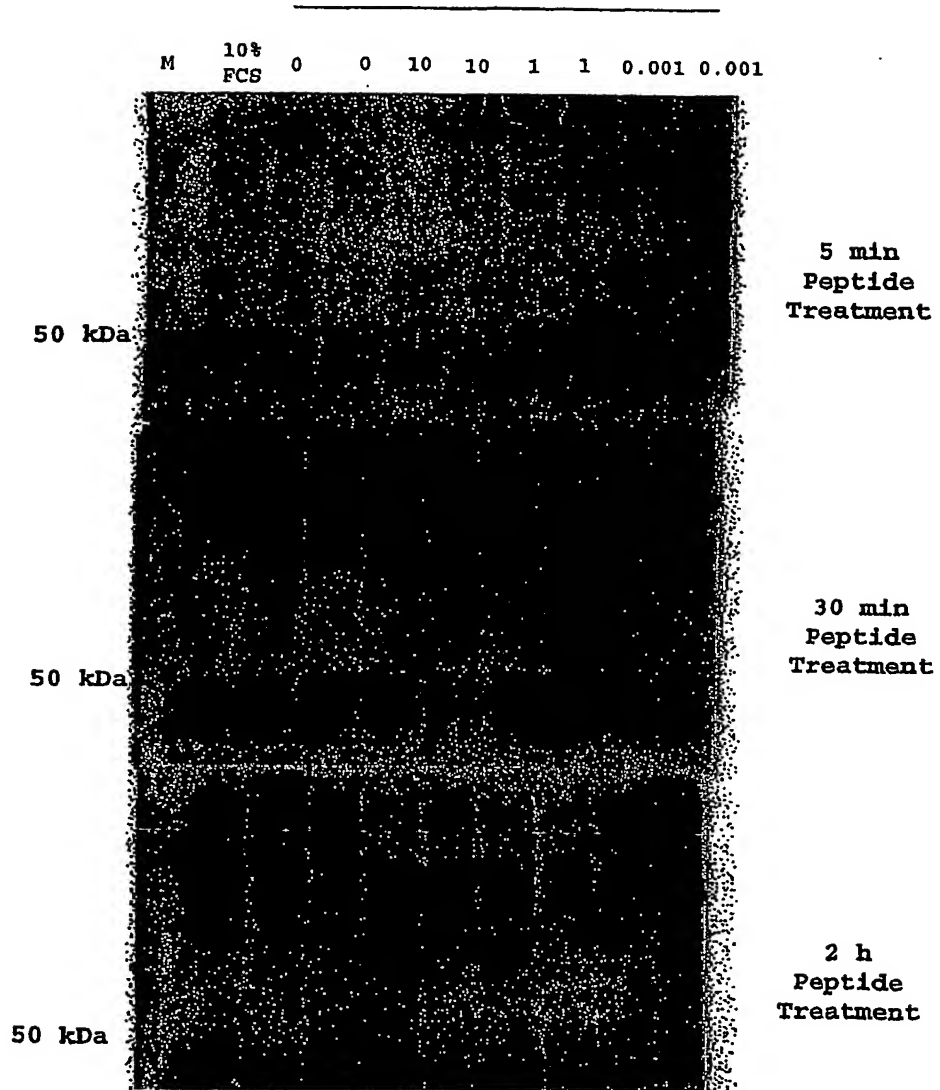
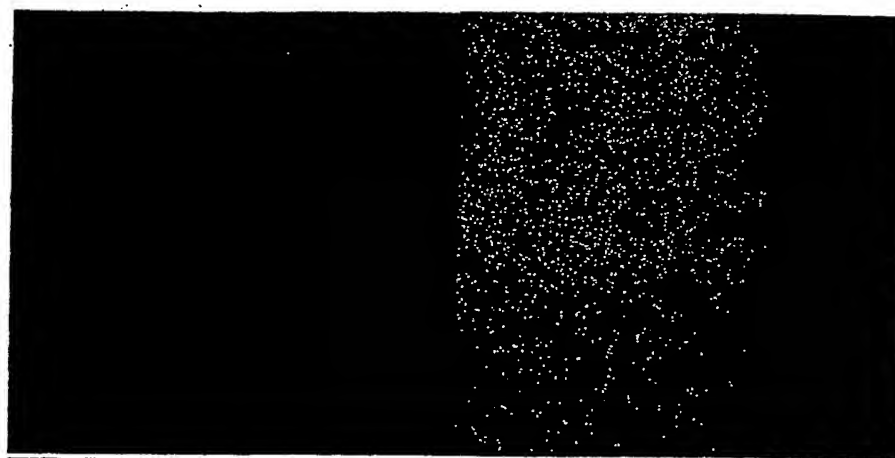


Figure 19b

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IRAP antibody staining

NeuN antibody staining



IRAP antibody staining

GLUT4 antibody staining

Figure 20

24/30

**Mitogenic actions of the AT₄ receptor :
[³H] Thymidine Incorporation**

• NG108-15

• SK-N-MC

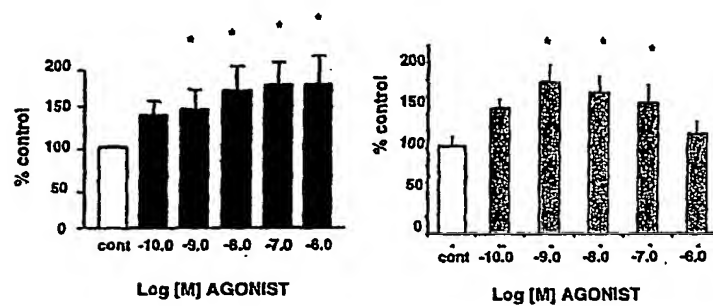


Figure 21

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**Effect of Ang IV and LVV-H7 on neurite outgrowth
in chick sympathetic neurones**

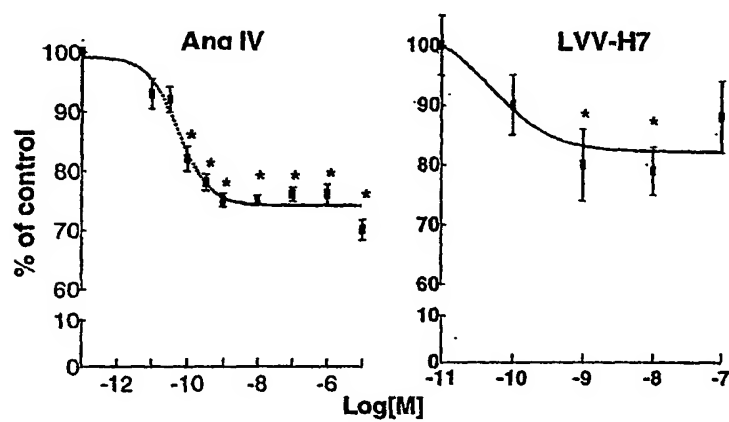


Figure 22

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**AT₄ receptor distribution in embryonic mouse brain :
sagittal sections**

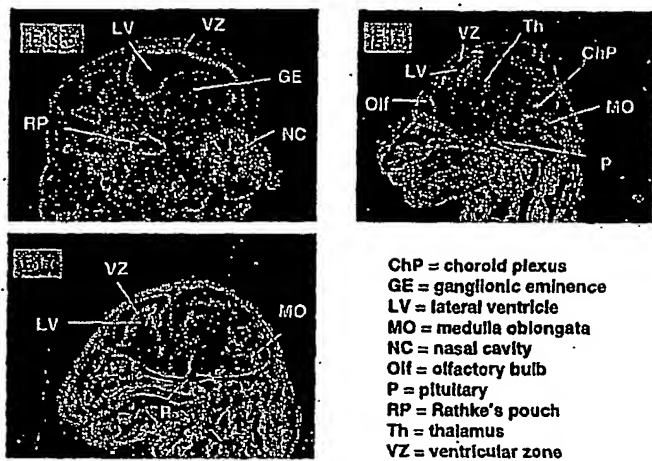


Figure 23

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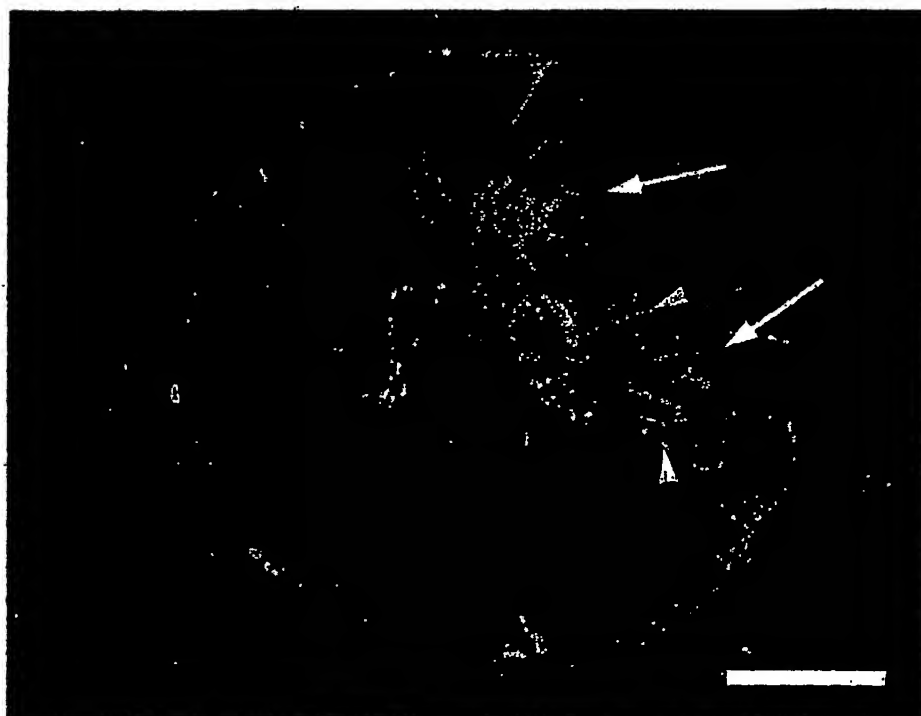


Figure 24

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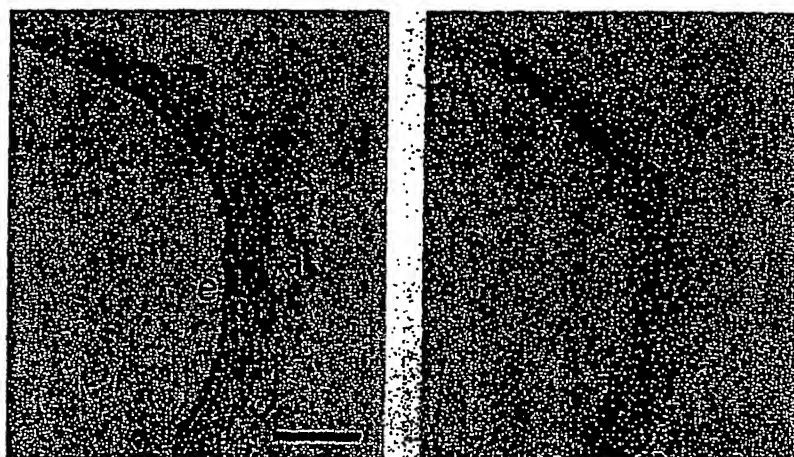
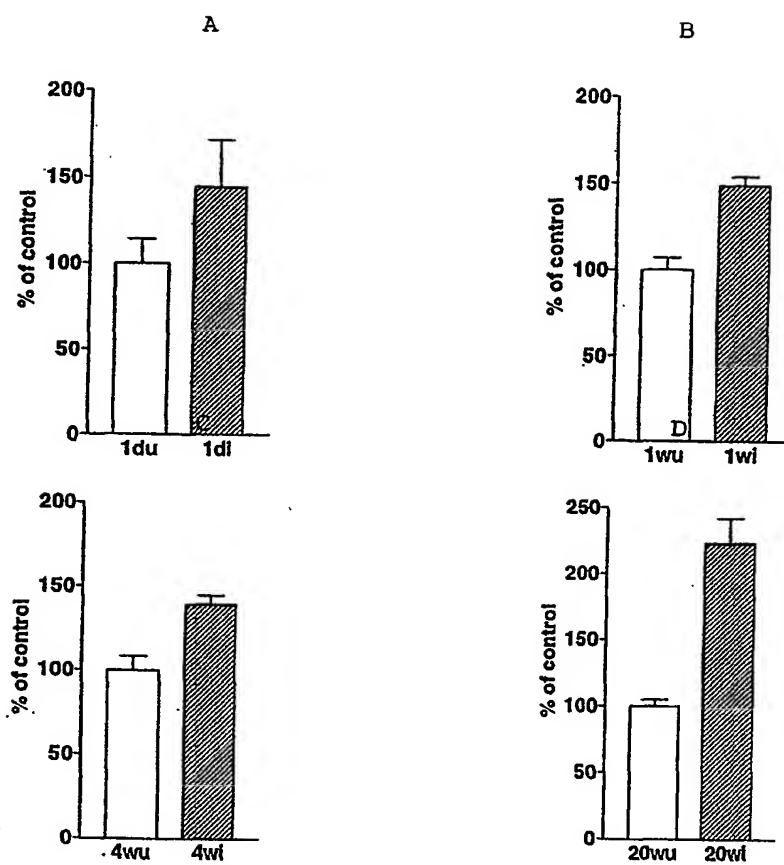


Figure 25

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*Figure 26*

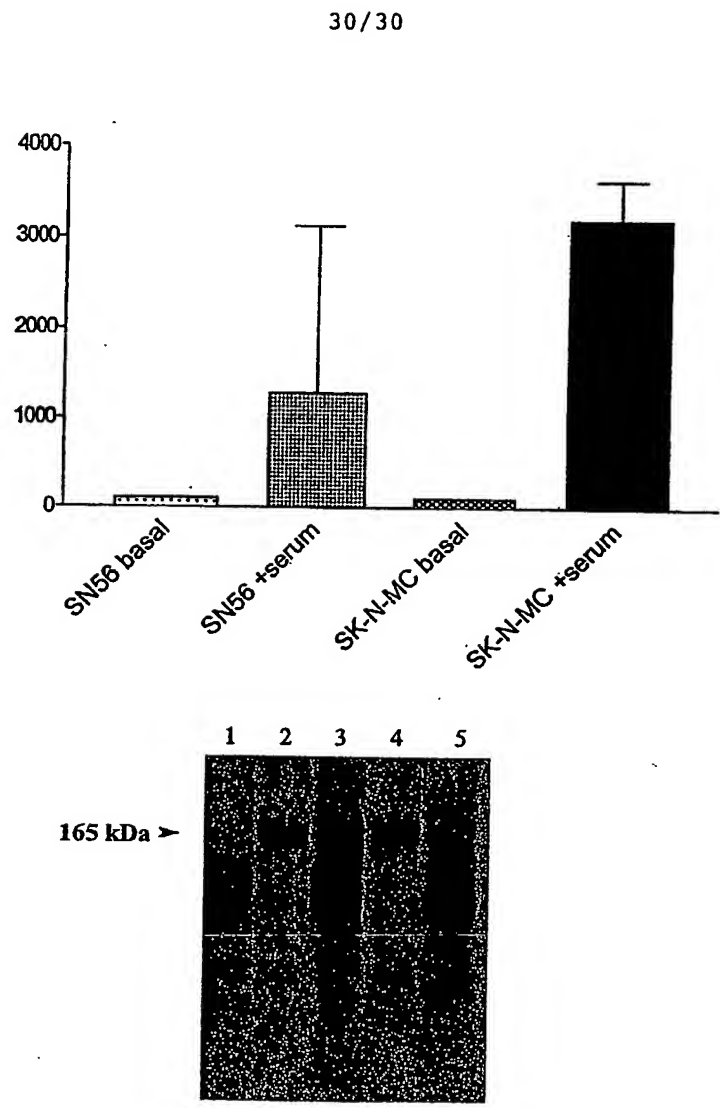


Figure 27

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU02/01034

A. CLASSIFICATION OF SUBJECT MATTER		
Int. Cl. ⁷ : A61K 31/713, 38/11, 38/55; A61P 3/00, 5/10, 25/00, 25/28, 25/30		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) IPC: ELECTRONIC DATABASE AS BELOW		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) DERWENT, MEDLINE: AT4, angiotensin IV receptor, cystinyl/cystine/cystyl-aminopeptidase, insulin-regulated membrane aminopeptidase, insulin-responsive aminopeptidase, otase, oxytocinase, placental leucine aminopeptidase, vasopressinase, vp165, IRAP, P-LAP		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98/01465 A1 (Howard Florey Institute of Experimental Physiology and Medicine) 15 January 1998. See the whole document.	1-7, 11-19, 21-25, 26-28, 34
X	EP 0838471 A1 (Sagami Chemical Research Center & Taisho Pharmaceutical Co LTD) 29 April 1998. See the whole document.	1-7, 11-19, 21, 23-25
X	EP 0882699 A1 (Taisho Pharmaceutical Co LTD & Sagami Chemical Research Center) 9 December 1998. See the whole document.	1-7, 11-19, 21, 23-25
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex		
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Date of the actual completion of the international search 10 September 2002		Date of mailing of the international search report 17 SEP 2002
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustalia.gov.au Facsimile No. (02) 6285 3929		Authorized officer G.J. MCNEICE Telephone No : (02) 6283 2055

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU02/01034

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94/00492 A1 (Washington State University Research Foundation) 6 January 1994. See the whole document.	1-28, 34
X	WO 00/77225 A1 (Whitehead Institute for Biomedical Research) 21 December 2000. See the whole document.	1-7, 11-19, 21, 23-25
X	WO 00/12544 A2 (Trustees of Boston University) 9 March 2000. See the whole document	1-7, 11-19, 21, 23-25
X	Derwent Abstract Accession No. 2001-441674/47, Class B04 D16 See whole document & WO 01/46413 A1 (Takeda Chemical Industries) 28 June 2001.	1-7, 11-19, 21, 23-25
P, X	Derwent Abstract Accession No. 2002-269344/31, Class B04 D16 See whole document & WO 02/16428 A1 (Takeda Chemical Industries) 28 February 2002.	1-7, 11-19, 21, 23-25
P, X	Albiston et al. (2001). Evidence that the angiotensin IV (AT ₄) receptor is the enzyme insulin-regulated aminopeptidase. The Journal of Biological Chemistry, Vol. 272, No. 52, Issue of December 28, pp. 48623-48626 (2001). See the whole document.	1-34
X	Herbst et al. (1997). Insulin stimulates cell surface aminopeptidase activity toward vasopressin in adipocytes. American Journal of Physiology, Vol. 272, pp E600-E606 (1997). See the whole document, particularly table 1.	1-7, 24, 25
X	Wright et al. (1999). Contributions of the brain angiotensin IV-AT ₄ receptor subtype system to spatial learning. The Journal of Neuroscience, May 15, 19(10):3952-3961 (1999). See the whole document.	1-7, 11-19, 21-25
X	Actions of newly discovered angiotensin peptides and receptors in brain [online], 5 May 1999. Retrieved on 10 September 2002 from <u>archived</u> URL: http://web.archive.org/web/19990505225323/http://www.hfi.unimelb.edu.au/research/neurochemistry/neurochemistry_detail.html See the whole document, particularly under the heading "Actions of newly discovered angiotensin peptides and receptors in brain".	1, 7, 24

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU02/01034

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, X	Rasmussen et al. (2000). Structure of the human oxytocinase/insulin-regulated aminopeptidase gene and localization to chromosome 5q21. European Journal of Biochemistry, Vol. 267, pp 2297-2306 (2000). See the whole document.	1, 11, 12, 29-34
Y, X	Applied Molecular Genetics (Book). R. L. Miesfield (Author). Copyright © 1999 by Wiley-Liss INC. ISBN 0-471-15676-0. See "Antisense Genetics", pages 181-183.	1, 11, 12, 29-34

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/01034

Box I Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos : 30-34
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 30-34 could not be searched effectively as sequence ID Nos 24-28 were not disclosed.

3. ☐ Claims Nos :
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU02/01034

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
WO	9801465	AU	33292/97	CA	2259137
		NZ	333632	EP	917535
EP	838471	AU	63179/96	CA	2226303
		WO	9703093	CN	1192746
EP	882699	AU	37090/97	CA	2242571
		WO	9805624	US	6066672
WO	9400492	AU	46492/93	EP	647239
		US	5854388	US	6022696
WO	200077225	AU	200054779		
WO	200012544	US	6141708		
WO	200146413	AU	200118946	JP	2001238685
WO	200216428	AU	200178777		
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